Human B Cells in Peripheral Tolerance

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Publications

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2. **Human IL-10-overexpressing B cells possess extensive regulatory capacity toward both innate and adaptive immune responses**
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3. **Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligonucleotides.**
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2 Summary

Tight regulation of immune responses is essential to prevent immune disorders such as allergies. A healthy response to innocuous environmental antigens is characterized by immunological tolerance while allergies are the result of an exaggerated immune response towards such antigens (also referred to as allergens). The exact mechanisms that determine whether an individual develops allergies are still not fully understood. Some of the key events that underlie the development of an allergic response are the differentiation and expansion of allergen-specific T helper (Th) 2 cells that mediate the activation of allergen-specific B cells, leading to the production of allergen-specific IgE antibodies. Healthy individuals that are exposed to an allergen frequently mount an active immune response without developing allergies. Such a response is characterized by the development of allergen-specific inducible interleukin (IL)-10-producing regulatory T (Tr1) cells. IL-10 is an immunosuppressive cytokine that inhibits antigen-specific T cell proliferation and cytokine production and is essential in peripheral tolerance to allergens, autoantigens and tumor antigens. Furthermore, IL-10 inhibits IgE production by B cells while augmenting the production of non-inflammatory IgG4 antibodies. Recent studies have identified IL-10-producing B (regulatory) cells as important regulators of immune responses primarily in autoimmune diseases.

This thesis is focused on the role of B cells in the regulation of immune responses, particularly in relation to induction and maintenance of peripheral tolerance to allergens. We characterized the phenotype and function of human inducible IL-10-producing regulatory B cells (Br1 cells) from peripheral blood (described in chapter 6.1). We identified Br1-related surface markers (high expression of CD25 and CD71 and low expression of CD73), demonstrated that Br1 cells potently suppress antigen-specific $CD4^+$ T cell proliferation and represent precursors of IgG4-producing plasma cells. We looked at the frequencies of allergen-specific Br1 cells from beevenom allergic patients before and after receiving allergen-specific immunotherapy (SIT), and from highly beevenom-exposed non-allergic beekeepers. We found that the

frequency of allergen-specific Br1 cells was low in allergic individuals and increased upon SIT to levels comparable to those observed in beekeepers. Next, we studied the effect of IL-10 overexpression on the function and phenotype of B cells (described in chapter 6.2). We found that IL-10 overexpression induces a regulatory phenotype in B cells, and arms them to suppress antigen-specific proliferation of PBMC, production of inflammatory cytokines and maturation and differentiation of monocyte-derived dendritic cells. Furthermore, IL-10-overexpressing B cells suppressed TLR2-L or TLR4-L-induced proinflammatory cytokines and chemokines from PBMC. Finally, we investigated activation of B cells by different TLR-ligands, cytokines, and CD40L stimulation and the modulation of these responses by mammalian suppressive telomeric oligodeoxynucleotide (ODN) sequences (described in chapter 6.3). We found that direct and indirect stimulation of human B cells through endosomal associated TLRs plays a pivotal role in polyclonal B cell activation and immunoglobulin production to maintain long-term serological memory in humans. Furthermore, we observed a direct and general suppressive effect of mammalian telomeric ODN on the activation of human B cells at the level of proliferation, class-switch recombination, plasma cell differentiation and immunoglobulin production. Suppression of B cell responses by telomeric ODN may represent a natural mechanism to control B cell activation under chronic inflammatory conditions.

The findings described in this thesis provide new insights into the role of B cells in the regulation of immune tolerance.

3 Zusammenfassung

Eine strikte Regulierung der Immunantwort ist notwendig um Erkrankungen wie Allergien zu verhindern, die durch eine Überreaktion des Immunsystems ausgelöst wird. Eine gesunde Immunantwort auf ungefährliche Antigene aus der Umwelt zeichnet sich durch immunologische Toleranz aus, während Allergien das Resultat einer übertrieben Immunantwort auf eben diese Antigene (auch Allergene genannt) sind. Die exakten Mechanismen welche entscheiden ob ein Individuum Allergien entwickelt, oder nicht, sind bis heute nicht vollständig verstanden. Eines der Schlüsselereignisse das zur Entstehung einer allergischen Immunantwort führt, ist die Differenzierung und Proliferation von allergenspezifischen T Helferzellen die wiederum die Aktivierung von allergenspezifischen B Zellen vermittelt. Dies führt zur Produktion von allergenspezifischen IgE Antikörpern. Gesunde Individuen, die regelmässig einem Allergen ausgesetzt sind, entwickeln eine Immunantwort gegen dieses Allergen ohne dabei Allergien zu entwickeln. Diese gesunde Immunantwort ist durch die Entwicklung von IL-10-produzierende, induzierbare T Zellen (Tr1) charakterisiert. IL-10 ist ein immunsuppressives Zytokin, das antigenspezifische T Zellproliferation und Zytokinproduktion unterdrückt und somit essentiell für die periphere Toleranz gegenüber Allergenen, Autoantigenen und Tumorantigenen ist. Ausserdem kann IL-10 die IgE-Produktion von B Zellen inhibieren, während es die Produktion der anti-inflammatorisch wirkenden IgG4 Antikörper erhöht. Kürzlich veröffentlichte Studien haben vornehmlich in Autoimmunerkrankungen IL-10-produzierende B Zellen (B regulatorische Zellen) als wichtige Regulatoren der Immunantwort identifiziert.

Diese Dissertation konzentriert sich auf die Rolle von B Zellen in der Regulation der Immunantwort, im Besonderen mit Bezug auf die Induktion und den Erhalt der peripheren Toleranz zu Allergenen. Wir haben den Phänotyp und die Funktion von humanen, induzierbaren, IL-10-produzierenden, regulatorischen B Zellen (Br1 Zellen) aus humanem, peripherem Blut charakterisiert (beschrieben in Kapitel 6.1). Wir haben Oberflächenmarker für Br1 Zellen identifiziert (hohe Expression von CD25

und CD71, niedrige Expression von CD73). Des weiteren haben wir gezeigt, dass Br1 Zellen effizient die Proliferation von antigenspezifischen CD4+ T Zellen unterdrückt und nachgewiesen, dass Br1 Zellen Vorläuferzellen von IgG4-produzierenden Plasmazellen sind. Bei der Betrachtung der Frequenz von Br1 Zellen in Bienengift-allergischen Patienten vor und nach spezifischer Immuntherapie und in häufig Bienengift ausgesetzten nicht-allergischen Imkern, fanden wir, dass die Anzahl von Br1 Zellen in allergischen Individuen niedrig ist. Durch spezifische Immuntherapie werden Br1 Zellen induziert und erreichen dabei ein Niveau vergleichbar mit denen von Imkern. Nächstfolgend haben wir den Effekt einer IL-10 Überexpression auf die Funktion und den Phänotyp von B Zellen unersucht (beschrieben in Kapitel 6.2). Wir konnten aufzeigen, dass eine IL-10-Überexpression einen regulatorischen Phänotyp in B Zellen induziert und sie mit der Fähigkeit ausrüstet antigenspezifische Proliferation von mononukleäre Zellen des peripheren Blutes und die Produktion von inflammatorischen Zytokinen zu unterdrücken. Zusätzlich wurde die Reifung und Differenzierung von aus Monozyten differenzierten Dendritische Zellen zu unterdrückt. IL-10-überexpermierende B Zellen können ausserdem die Zytokin- und Chemokinproduktion der mononukleären Zellen des peripheren Blutes nach TLR2- und TLR4-Stimulation unterdrücken. Schlussendlich haben wir die Aktivierung von B Zellen nach Stimulation mit unterschiedlichen TLR-Liganden, Zytokine und CD40-Ligand untersucht und die Modulation dieser Aktivierung durch suppressive Telomersequenzen von Säugetieren analysiert (beschrieben in Kapitel 6.3). Wir fanden heraus, dass die direkte und indirekte Stimulation von humanen B Zellen durch endosomal-assoziierte TLRs ein Schlüsselrolle in der polyklonalen B Zellaktivierung und Immunglobulinproduktion spielen und diese Stimulation notwendig ist um ein langfristiges serologisches Gedächtnis im Menschen aufrechtzuhalten. Ausserdem haben wir einen direkten und generalisierten suppressiven Effekt durch Oligodinukleotide aus Telomeren von Säugetieren auf die Aktivierung von B Zellen beobachtet. Sie unterdrückten die Proliferation, den Isotypenklassenwechsel, Plasmazellendifferenzierung und Immunglobulinproduktion. Unterdrückung der B Zellantwort durch telomerische Oligodinukleotide könnte einen natürlichen Mechanismus

darstellen, der die B Zellaktivierung unter entzündlichen Bedingungen kontrolliert.

Die Ergebnisse, die in dieser Dissertation beschrieben werden, geben neue Einsichten in die Rolle von B Zellen in der Regulation der Immuntoleranz.

4 Abbreviations

AID/AICDA	Activation-Induced (Cytidine) Deaminase
APC	antigen-presenting cell
APRIL	a proliferation-induced ligand
B10	IL-10-competent B cell
BAFF	B-cell activating factor of the TNF-family
BAFF-R	BAFF receptor
B10pro	progenitor B10
BCR	B cell receptor
BCMA	B cell maturation antigen
Be	B effector
BSA	bovine serum albumin
CIA	collagen-induced arthritis
C_H	immunoglobulin heavy chain constant
CHS	contact hypersensitivity
C_V	immunoglobulin heavy chain variable
CSR	class switch recombination
CTLA-4	cytotoxic T lymphocyte antigen 4
D	immunoglobulin heavy chain diversity region
DAMP	danger-associated molecular pattern
DC	dendritic cell
DTH	delayed-type hypersensitivity
ds	double stranded
EAE	experimental autoimmune encephalomyelitis
GARP	glycoprotein A repetitions predominant
HR	histamine receptor
ICOS	inducible T cell costimulator
Ig:	immunoglobulin
IGH@	immunoglobulin heavy locus
IGK@	immunoglobulin kappa locus
IGL@	immunoglobulin lambda locus
ILC	innate lymphoid cell
IL-$10^{-/-}$ B cell	chimeric mice specifically lacking IL-10-producing B cells
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked
iTreg	induced Treg
J_H	immunoglobulin heavy chain joining region
L	ligand
LAT	linker for activation of T cells
LPS	lipopolysaccharide
LYN	tyrosine-protein kinase Lyn
mAb	monoclonal antibody
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MZ	marginal zone
NK	natural killer
nTreg	natural Treg

NOD	nucleotide-binding oligomerization domain
ODN	oligodeoxynucleotide
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
pDC	plasmacytoid dendritic cell
PRR	pattern recognition receptor
RAG	Recombination activating gene
RIG-I	retinoic acid-inducible gene 1
sIgA	secretory IgA
SIT	specific immunotherapy
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
ss	single stranded
TACI	Transmembrane activator and cyclophilin ligand interactor
TIR	Toll/IL-1R homology
TIRAP	TIR domain containing adaptor protein
T2-MZP	transitional 2 marginal zone precursor
TCR	T cell receptor
TD	thymus-dependent
T_{FH}	T follicular helper
TLR	toll-like receptor
TNP	trinitrophenyl
TRAF	TNF receptor associated factors
TRAM	TLR4 adaptor protein
Treg	regulatory T cell
TRIF	TIR-domain-containing adaptor-inducing IFN-β
Th	T helper
TI	thymus independent
V_H	immunoglobulin heavy chain variable region
wt	wild type

5 General introduction

5.1 History

The earliest documented cases of allergies date back to ancient times. King Menes of Egypt who lived around 3000 BC, died after a wasp sting. In the Roman Empire, several members of the Julio-Claudian imperial house suffered from atopic diseases. Emperor August suffered from seasonal rhinitis and pruritic skin disease. Claudius suffered from rhinoconjunctivitis and his son Brittanicus was allergic to horse dander.

In 1809 John Bostock was the first to accurately describe hay-fever as a disease that affected the upper respiratory tract.[1] Later, in 1873, Charles Blackely identified pollen as causative agent for allergic reactions.[2] In 1906, Clemens von Pirquet introduced the term 'allergy' (Greek *allos,* other; *ergon*, reaction), to describe the phenomenon that patients who had previously received horse serum or smallpox vaccine showed an altered reaction to a subsequent application.[3] This initial description of an allergic reaction included protective immunity as well as hypersensitivity and basically relates to the concept of immunological memory.

In the late 1900s, von Behring and Kitasato experimented with tetanus and diphtheria toxins. They immunized animals with cell-free filtrates of these pathogens and demonstrated that the serum from these immunized animals could protect a naïve recipient from developing disease. They proposed a 'lock and key' interaction between receptors (of immune cells) and specific toxins (derived from pathogens) to be the trigger for antibody production.[4] This lock and key principle was confirmed by Pauling in 1940.[5] Heidelberger and Avery demonstrated that antibodies were proteins that could precipitate when antigens were added.[6] In 1921 Carl W. Prausnitz and Hans Küster demonstrated that reactivity to an allergen could be transferred by intradermal injection of serum from a sensitized individual to a healthy recipient.[7] In the late 1960s, IgE was discovered independently by two different groups: Kimishige and Teruko Ishizaka[8] and Hans Bennich and S.G.O Johansson.[9]

Currently, the term allergy is used synonymously with IgE-mediated allergic disease and represents the first type of four classical hypersensitivity responses as proposed by Coombs and Gell in 1963.[10]

5.2 The immune system

The immune system can be divided into two distinct, but highly interactive components: innate and adaptive immunity. The principal difference between the two is that innate immunity is germline-encoded, while a key feature of adaptive immunity is the rearrangement and mutation of the genes encoding B cell receptors (BCR) and T cell receptors (TCR) resulting in a broad repertoire of receptors, each with its own unique structure and resulting antigen-specificity. Innate immunity provides quick and general protection against invading microbes. The key components that make up the innate immune system are the skin, lung and intestinal epithelium, mucosal layers and other physical barriers. The major innate immune cell types are monocytes, macrophages, dendritic cells (DCs), granulocytes, megakaryocytes and innate lymphoid cells (ILCs). ILCs form a recently described family of innate cells consisting of ILC1, ILC2, ILC3, LTi and natural killer (NK) cells. NK cells have been known for many years already and can be considered the founding members of the ILC family.[11] Adaptive immune responses to newly encountered antigens require a relatively long time to develop compared to innate responses. The main cell types of the adaptive immune system are B and T lymphocytes.

5.2.1 Innate immune system

Cells and tissues of the innate immune system

The innate immune system provides a first line of defense against invading pathogens. It involves (I) physical barriers that are formed by the cornified layers and keratinocytes of the skin as well as the epithelial and mucosal layers of the respiratory, urogenital and gastrointestinal tracts; (II) Soluble factors including antimicrobial peptides, lysozyme and chemokines as well as acute phase proteins such as C-reactive protein, anti-bacterial proteins such as mannose-binding lectin and finally the complement system, which consists

of a complex system of plasma proteins that can be activated through different pathways and ultimately leads to lysis of microbes; (III) Cytokines play a key role in the regulation of innate immune cells as well as in the crosstalk between the innate and adaptive immune responses;[12] (IV) Immune cells including phagocytic cells (macrophages, neutrophils and DCs), granulocytes (basophils and eosinophils), mast cells, and NK cells. Some immune cell types cannot be easily classified as a member of the innate or adaptive immune system. The recently described family of ILCs fits the definition of innate immune cells as cells that do not have genetic receptor rearrangements. However, these cells appear to require priming phases to be fully functional and therefore may not be a typical first line of defense quick responder cell type.[11] Splenic marginal zone B cells and γδ T cells may also be regarded as innate-like immune cells, since they can mount rapid humoral responses against blood-borne antigens and do not frequently have mutated antigen receptor genes.[13, 14]

Receptors of the innate immune system

Many innate immune cells express germline-encoded pattern recognition receptors (PRRs). The main groups of PRRs are Toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors. These receptors recognize a broad range of conserved pathogen-associated molecular patterns (PAMPs). These PAMPs are typically molecules that are not expressed by mammalian cells but are essential for survival or proliferation of pathogens. Recent studies have demonstrated that certain PRRs also recognize endogenous molecules that are released by damaged cells. These endogenous PRR-activating molecules are also referred to as danger-associated molecular patterns (DAMPs). Most PRRs upregulate expression of genes that mediate inflammatory responses including pro-inflammatory cytokines like type I interferons, IL-1, IL-6 and TNF as well as chemokines and antimicrobial proteins.[15]

TLRs form the best-characterized PRR family and they recognize a broad range of pathogens including bacteria, viruses, parasites, fungi and protozoa. All TLRs consist of an N-terminal leucine-rich repeat domain, a transmembrane region and an intracellular Toll/IL-1R homology (TIR) domain. So far, 10 human and 13 murine TLRs have been described. Here I will focus on the human TLRs. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell membrane while TLR3, TLR7, TRL8, TLR9 and TLR10 are found in endolysosomes. Each TLR recognizes a distinct group of microbial (and in some cases host-derived) ligands and some TLRs function as heterodimers (listed in table 1 and illustrated in figure 1). TLR1 forms dimers with TLR2 and recognizes bacterial triacylated lipoproteins, TLR2 alone recognizes lipoproteins from varying origin, TLR3 recognizes viral double stranded RNA (dsRNA) and TLR4 recognizes bacterial lypopolysaccharide (LPS). Binding of LPS to TLR4 requires interaction with CD14 and the adaptor protein MD2. TLR5 recognizes bacterial flagellin. TLR6 forms functional heterodimers with TLR2, which recognize bacterial and viral diacylated lipoproteins, TLR7 and 8 recognize single stranded RNA (ssRNA), which can be of viral, bacterial or host origin. TLR9 recognizes unmehtylated DNA with CpG motifs, which are primarily from microbial origin. In humans TLR9 is mainly expressed by B cells and plasmacytoid DCs (pDCs).[16] The optimal ligand for activating human TLR9 consists of unmethylated CpG dinucleotides and a TCGTT and/or TCGTA motif in its flanking region. Three major classes of CpG ODNs have been described: A-class (also known as D-type ODNs), B-class (also known as K-type ODNs) and C-class. A-class CpG ODNs consist of a mixed phosphodiester–phosphorothioate backbone and contain a hexameric purine–pyrimidine–CG–purine–pyrimidine motif flanked by self-complementary bases and a poly-G tail at its 3' end. A-class CpG ODNs potently activate human pDC to produce IFN-α.[17] B-class CpG ODNs have multiple CpG motifs on a phosphorothioate backbone and strongly induce proliferation and activation of B cells.[18, 19] C-class CpG ODNs have structural features of both A- and B-class CpG ODNs as they typically consist of a phosphorothioate backbone containing a 5' TCGTCG sequence (similar to B-class CpG ODNs) and have palindromic sequences combined with stimulatory CpG motifs (similar to A-

class CpG ODNs) but no poly G motif. C-class CpG ODNs can activate both human pDCs and B cells.[20] All three classes of CpG ODNs depend on TLR9 for signaling. Under certain conditions TLR9 also recognizes self DNA which can lead to autoimmune diseases like systemic lupus erythematosis (SLE).[21] The ligand for TLR10 remains unknown. All nucleotide-binding TLRs are localized in endolysosomes. This compartmentalization prevents unwanted chronic activation by host cell-derived nucleotides.[15]

With the exception of TLR3, all TLRs can signal through the adapter molecule MyD88. TLR1/2 and TLR2/6 signal through TIR domain containing adaptor protein (TIRAP), which is alternatively named MyD88 adapter-like (MAL) and MyD88, while TLR3 does not use MyD88 but signals through a different adapter molecule called TIR-domain-containing adaptor-inducing IFN-β (TRIF). TLR4 utilizes TIRAP/MAL and MyD88 but can also signal through TLR4 adaptor protein (TRAM) and TRIF. TLR5, 7, 8 and 9 can only signal through MyD88. Activation of MyD88 is followed by recruitment of IRAK1/4 and TRAF6 ultimately leading to the activation of NF-κB, MAPK and AP-1 and production of inflammatory cytokines. TLR7/8 and TLR9 signaling through MyD88 and IRAK4 induces, in addition to NF-κB-dependent cytokine production, activation of IRF7 and ultimately production of type I interferons (illustrated in figure 1).[15] The ultimate outcome of TLR signaling in terms of cellular responses, including cytokine production, strongly depends on the cell type. DCs respond to TLR stimulation by upregulation of major histocompatibility complex (MHC) and costimulatory molecules as well as production of proinflammatory cytokines including TNF-α, IL-1, IL-6 and Th1-inducing cytokines IL-12 and IL-18. Lack of TLR signaling as demonstrated in MyD88-deficient mice leads to enhanced Th2 responses and consequently elevated IgE production.[22] In the following section the focus will lay on the effect of TLR signaling on human B cells.

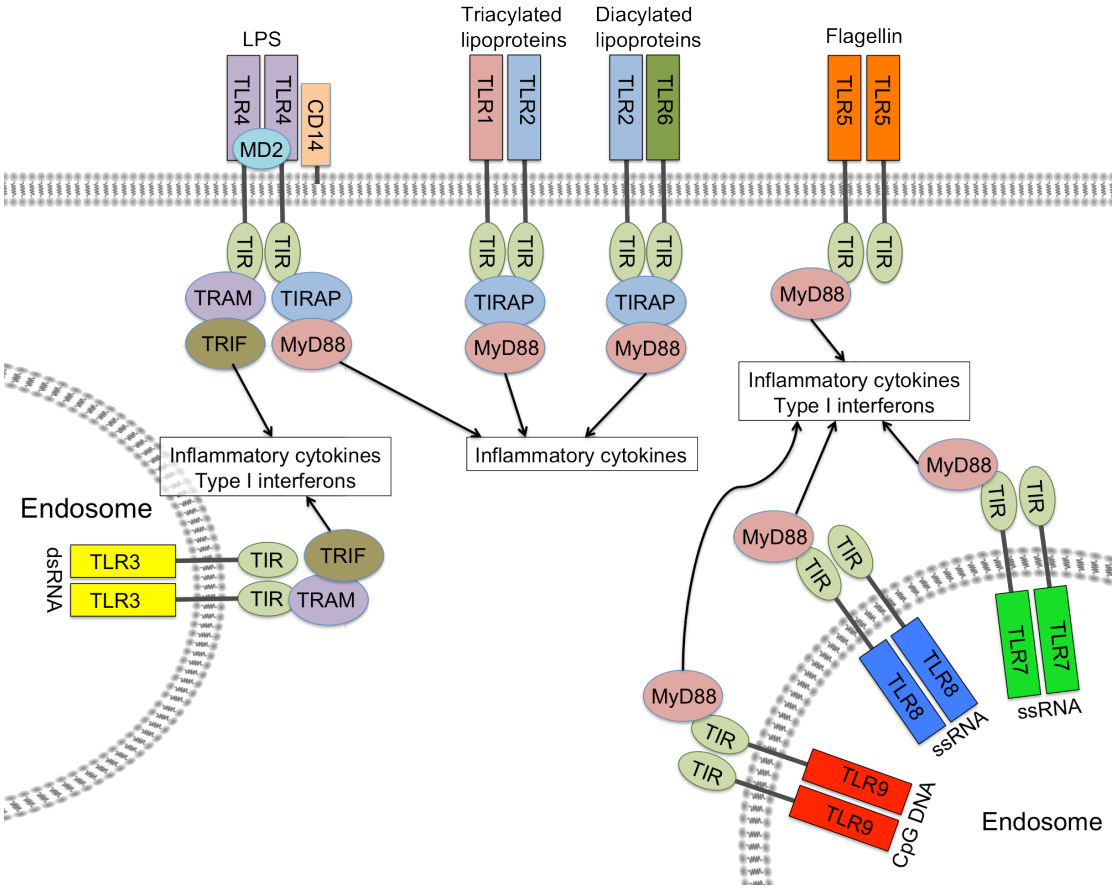

Figure 1. Ligands, cellular localization and initiation of signaling of TLRs. TLR1, TLR2, TLR5 and TLR6 are located on the surface membrane, while TLR3, TLR7, TLR8 and TLR9 are located on endosomal membranes. TLR1/2 heterodimers recognize triacylated bacterial lipoproteins, while TLR2/6 heterodimers bind diacilylated lipoproteins. Both heterodimers signal through TIRAP and MyD88. TLR3 recognizes dsRNA and signals through TRAM and TRIF. TLR7 and TLR8 recognize ssRNA and both signal through MyD88. TLR9 recognizes hypomethylated CpG DNA and also signals through MyD88.

TLRs in B cell responses

Human peripheral B cells express TLR1, TLR6, TLR7, TLR8, TLR9 and TLR10. These TLRs can be detected in resting naïve B cells and are strongly upregulated upon B cell activation in response to BCR or CD40 ligation and exposure to CpG ODN. Circulating memory B cells also show higher expression of these TLRs than naïve B cells. TLR9 and TLR10 are among the strongest expressed TLRs on B cells. Murine and human B cells have a similar expression profile of TLRs, with the exception that human B cells do

not express TLR4 while this receptor is readily detected in murine resting B cells.[23]

TLR signaling provides a third signal in addition to antigen (through BCR signaling) and T cell help (through CD40 ligation) that is required for efficient B cell proliferation, isotype switch and plasma cell differentiation. Pasare and Medzitov showed that mice that specifically lacked MyD88 in B cells mounted less efficient antibody responses to thymus-dependent (TD) antigens.[24] The expression of TLRs is strongly elevated in activated and memory B cells. As a result, these cells respond much stronger to TLR signaling (which reflects microbial exposure) and may be triggered by TLR signaling to differentiate to immunoglobulin-producing plasma cells while naïve cells first require antigen-specific activation.[25] TLR signaling can also lead to cytokine production in B cells as will be discussed in the section concerning regulatory B cells. The role of TLR signaling in the regulation of class-switch recombination (CSR) will be discussed below in the section concerning CSR.

Table 1. TLRs, their cellular localization and ligands. (Adapted from[15])

TLR	Localization	Ligand	Origin of the Ligand
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Viruses
TLR4	Plasma membrane	LPS	Bacteria
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TRL7/8	Endolysosome	ssRNA	Viruses, bacteria, self
TLR9	Endolysosome	CpG-DNA	Viruses, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown

5.2.2 Adaptive immune system

T and B lymphocytes are the cells that form the core of the adaptive immune system. They express highly specific and diverse antigen-specific cell-surface receptors. Furthermore these cells are unique in their capacity to generate a memory compartment following initial antigen exposure. This allows rapid clearance of pathogens upon a secondary exposure. The two pillars of the

adaptive system response are humoral (mediated by B cells and immunoglobulins) and cell-mediated immunity (mediated by T cells). This section outlines the basic mechanisms that define adaptive immune responses with an emphasis on B cells and humoral immunity.

Generation of receptor diversity in T and B lymphocytes

Both T and B cells have surface-membrane-associated receptors that enable specific recognition of antigens: TCR and BCR. An intriguing feature of T and B cells is the wide range of antigens that are specifically recognized by their surface receptors. The variety of different antigen-specific TCR and BCR expressed by T and B lymphocytes respectively is much larger than can be expected based on the size of the genome. Below I will describe the mechanism that generates this tremendous diversity of antigen-specific receptors in B cells. TCR diversity is generated in a similar manner but will not be discussed here.

The genes encoding the human BCR heavy and light chains are located in the immunoglobulin heavy locus (IGH@) on chromosome 14 and the immunoglobulin light chain lambda (IGL@) and kappa (IGK@) loci on chromosomes 22 and 2 respectively. The germline IGH@ locus contains >100 functional variable (V) gene segments, 27 diversity (D) gene segments and 9 joining (J) segments. Both light chain loci contain 30-35 V segments and 4-5 J segments while D segments are absent from light chain loci. The key mechanism that is required for the generation of receptor diversity is a process called V(D)J recombination. In order for a B lymphocyte to express a functional BCR, it needs to rearrange the germline V(D)J genes in such a way that one V, one D (only for IGH@) and one J segment are selected and joined to form a new V(D)J exon (This process is illustrated for the IgH chain locus in figure 2). This is the first step in generating receptor diversity. A second layer of diversification is added through removal or addition of nucleotides at the junctions between the V and D, the D and J, or the V and J segments.[26]

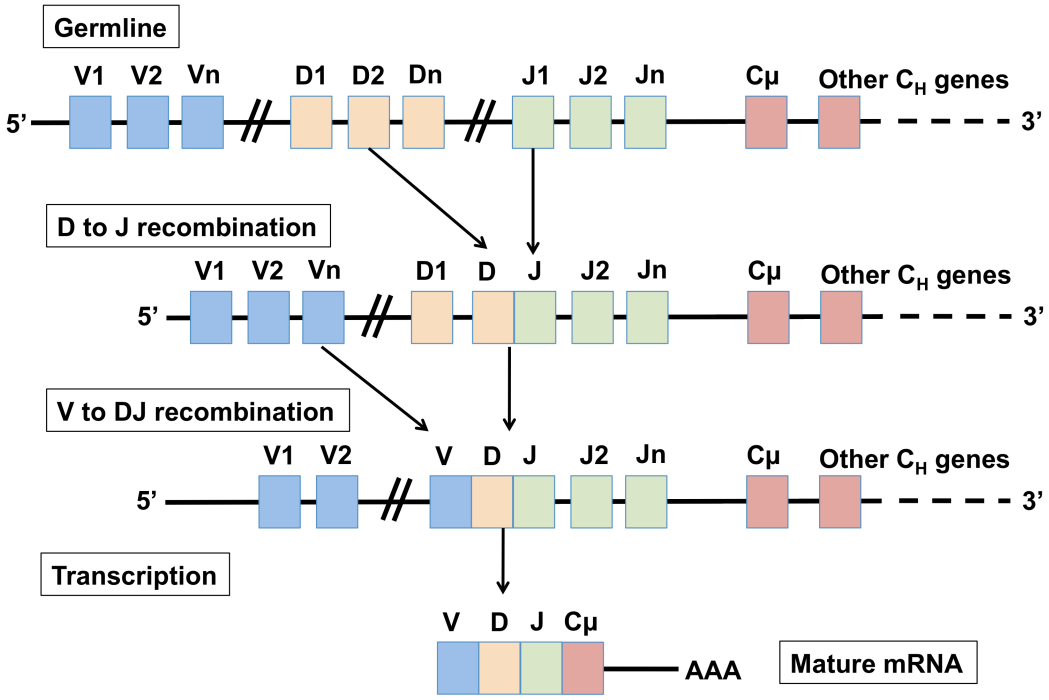

Figure 2. V(D)J recombination of the immunoglobulin heavy chain locus. Germline DNA contains an array of different V, D and J genes. During the process of V(D)J recombination one V, one D and one J gene are fused to form a recombined V(D)J gene. First a single D and a single J gene recombine and any intervening DNA is excised and deleted from the chromosome. Then V to DJ recombination takes place resulting in the formation of a rearranged VDJ gene. The mature mRNA transcript that is generated from this sequence contains the rearranged VDJ segment together with the constant region of the IgH chain (Cμ or Cδ in mature naïve cells) and translates into an IgM or IgD immunoglobulin heavy chain protein. (Adapted from[27]).

T lymphocytes

T cells mature in the thymus where the cells that strongly recognize self-antigens presented by thymic epithelial cells are eliminated to prevent the maturation of autoreactive T cells. Furthermore, T cells are positively selected for their capacity to interact with MHC class I or MHC class II molecules. During this stage the fate of the T cell to become a $CD4^+$ or a $CD8^+$ T cell is determined. Thymus-selected T cells are released into the circulation as mature naïve T cells and can be activated when they are presented with their cognate antigen in the context of MHC class I (for $CD8^+$ T cells) or MHC class II (for $CD4^+$ T cells). $CD8^+$ T cells are cytotoxic cells that can lyse virus-

infected host cells and tumor cells. $CD4^+$ T cells are also referred to as helper T cells because they help other cells to carry out their effector function.

CD4+ T cells

Naïve $CD4^+$ T cells can differentiate into different types of effector T cells depending on the context in which they are activated. The best-characterized Th cell subsets are Th1, Th2, Th17, natural (n) regulatory T cells (Treg) and induced (i)Treg cells. DCs are essential during this process. DCs capture antigens, migrate to draining lymph nodes and present antigenic peptides on MHC class II molecules to naïve T cells. DCs are activated and mature in response to PRR signaling as a result of exposure to PAMPs. The cytokines that are secreted by DCs during their interaction with antigen-specific naïve $CD4^+$ T cells are crucial for the fate of these T cells. If IL-4 is present at this stage, $CD4^+$ T cells mainly differentiate to Th2 cells, which secrete IL-4, IL-5 and IL-13.[28] IL-12 induces IFN-γ-producing Th1 cells while a combination of IL-6, TGF-β and IL-1β induces Th17 cells, which produce IL-17A, IL17F and IL-22.[29] Furthermore, some less clearly defined Th subsets have been described including IL-9-producing Th9 cells, IL-10-producing Tr1 cells, TGF-β-producing Th3 cells, and IL-21-producing T follicular helper (Tfh) cells and IL-22-producing Th22 cells.[30, 31] Most of these Th cell subset have a master transcriptional regulator that drives the differentiation program. These transcription factors are induced by APC-derived cytokines that initiate the differentiation of naïve T cells and the following are generally accepted: Tbet for Th1 cells, GATA3 for Th2 cells, RORγt for Th17 cells, Bcl6 for Tfh cells.[31] PU.1 has been proposed as the master regulator for Th9 cells.[32] nTregs and iTregs express FoxP3.[33] A master regulator for Tr1, Th3 and Th22 cells remains to be identified.

Regulatory T cells

Regulatory T cells are key factors in the maintenance of peripheral tolerance. There are two major types of regulatory T cells: nTreg and iTreg cells.

nTregs are characterized by the expression of high levels of the IL-2Rα chain (CD25) and the transcription factor FoxP3. The majority of nTregs is generated in the thymus as antigen-primed and mature T cells. Under certain conditions nTregs can develop in the periphery from conventional naïve T cells.[34] Their main function is to suppress effector cells at sites of inflammation. nTregs strongly suppress proliferation of both naïve and memory $CD4^+$ T cells.[35] FoxP3 is critical for the development of nTreg cells and human mutations in the *FOXP3* gene lead to dysfunctional Tregs culminating in immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, which is characterized by the development of autoimmune diseases, inflammatory bowel disease and allergies.[36] activated nTreg cells produce immunosuppressive cytokines including IL-10 and TGF-β. Interestingly, the suppressive function of nTregs does not critically depend on these cytokines.[37] nTreg cells express cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) on their surface membrane. Both molecules belong to the CD28 family of co-stimulatory receptors and negatively regulate signaling in activated T cells. Another nTreg-associated surface marker is glucocorticoid-induced tumor necrosis factor receptor (GITR). Stimulation of GITR inhibits nTreg-mediated suppression through breaking the anergic state of nTreg cells.[38, 39] The frequency of nTregs in human PBMC is approximately 3%.

Induced Treg cells are suppressor cells that develop in the periphery from conventional $CD4^+CD25^-$ T cells. These cells exert their function primarily in a contact-independent manner through secretion of suppressive cytokines such as IL-10 and TGF-β.[40] There are two major types of induced Treg cells: Tr1 and Th3 cells.

Tr1 cells can be generated from naïve or resting $CD4^+$ T cells. This was first demonstrated by stimulation of naïve T cells from OVA TCR transgenic mice (all T cells are specific for OVA in this model) with IL-10 and OVA. This led to the differentiation of a Th cell subset with a cytokine profile that was different from Th1 or Th2 cells and included IL-10, IL-5 and IFN-γ production in

absence or presence of TGF-β.[41] Like nTreg cells, Tr1 cells constitutively express CTLA-4, while a subset of Tr1 cells expresses PD-1. Tr1 cells can suppress the activation of naïve and memory T cells, Th1 and Th2 responses and the antigen-presentation capacity of DCs. Tr1-mediated suppressive mechanisms critically depend on IL-10.[40]

Th3 cells were initially identified in an experimental autoimmune encephalomyelitis (EAE) model. Mice were orally exposed to an autoantigen (myelin base protein) to which autoreactive T cells were generated. The majority of myelin base protein-specific $CD4^+$ T cells secreted TFG-β and protected against EAE.[42] Th3 cell-mediated immune suppression is antigen-nonspecific and primarily mediated through secretion of TGF-β.[40]

B lymphocytes

B cells are responsible for the generation of humoral immune responses. These cells are the precursors of immunoglobulin-producing plasma cells. B cell develop in the bone marrow. Like all myeloid and lymphoid cells, B cells differentiate from hematopoietic stem cells. The earliest B cell-specific precursors are called pre-pro B cells. Pre-pro B cells still have germline immunoglobulin heavy and light chain genes and do not express CD19 or BCR on their surface membrane. These cells express the IL-7 receptor (IL-7 is essential for proliferation of B cell precursor cells) and downregulate c-kit expression (which is expressed at earlier developmental stages). The expression of recombination activating genes (*RAG*)*1* and *RAG2* and terminal deoxynucleotidyl transferase (*TdT*) are upregulated at this stage. These genes encode enzymes that are essential for the process of V(D)J recombination. The next B cell precursor stage is the pro-B cell stage. At this stage expression of B cell lineage-specific transcription factors including Paired box protein-5 PAX5 and early B-cell factor 1 (EBF1) is strongly upregulated. Furthermore, surface expression of the B cell lineage marker CD19 and the BCR-associated adaptor proteins CD79α and CD79β, which mediate BCR signaling, is upregulated. Transition from pro B cells to early Pre B cells is characterized by surface expression of IgM with a rearranged VDJ

on its IgH chain. The Ig light chain has not rearranged at this stage and surface IgM heavy chains are linked to so-called surrogate light chains. Early Pre B cells that fail to express surface IgM with surrogate light chains will not progress to the late pre B cell stage and are eliminated. Late Pre B cells undergo VJ recombination of their Ig light chain genes and then progress to the immature B cell stage expressing surface IgM consisting of rearranged IgH and Ig light chains. If an immature B cell recognizes self antigens it can either become anergic (non-responsive) or undergo another round of V(D)J recombination (this process is referred to as receptor editing) to avoid the generation of autoreactive B cells. Non-autoreactive immature B cells are released into the circulation and migrate to the spleen where they undergo further maturation. Mature B cells can be roughly divided into two subsets: conventional follicular B cells (frequently referred to as B-2 cells) and non-conventional extrafollicular B cells (including B-1 cells, which have been mainly characterized in the mouse and marginal zone (MZ) B cells).[43, 44]

B cell responses to thymus-dependent antigens

Follicular B cells participate in TD responses. These cells interact with helper T cells in germinal centers that are mainly found in secondary lymphoid organs. B cell activation to T-cell dependent antigens requires BCR stimulation and CD40 ligation. Antigens can be captured directly by B cells or can be presented by follicular DCs in the lymphoid follicles. BCR stimulation is typically mediated through binding of a specific antigen to the BCR leading to internalization, processing and presentation of antigenic peptides in MHC class II molecules. Antigen can be presented in this way to $CD4^+$ T cells, which are activated in this manner. B cells are believed to be poor activators of naïve T cells and primarily interact with effector memory $CD4^+$ T cells. Activated $CD4^+$ T cells upregulate CD40L and secrete cytokines. The type of cytokines that are produced by these T cells depends on how these cells were primed as naïve T cells. CD40L binds to CD40 on the B cell. CD40-CD40L interaction and the local cytokine milieu provide the second signal that is required for efficient B cell activation including proliferation, CSR and plasma cell differentiation.

Recently a subset of $CD4^+$ helper T cells was described that specializes in B cell help during germinal center reactions. These cells are called Tfh cells. The master regulator transcription factor for Tfh cells is Bcl-6 and Tfh cells express high levels of CXCR5, PD-1 and inducible T cell costimulator (ICOS). Due to their expression of CXCR5, these cells migrate towards CXCL13, which is found in B cell follicles of germinal centers. The signature cytokine of T_{FH} cells is IL-21, which induces strong B cell proliferation and promotes CSR and plasma cell differentiation.[45] Besides IL-21, T_{FH} cells can produce IL-4, IFN-γ and IL-10. To date, the developmental relation between Tfh cells and other helper T cells subsets remains controversial. Tfh cells may represent an independent lineage of specialized cells that aid B cells during germinal center reactions but they might also derive from other differentiated Th cell subsets.[46] Nevertheless, it is clear that these cells are essential for efficient germinal center reactions involving follicular B cells.

Follicular B cells that receive T cell help (through CD40L and cytokines) will become germinal center B cells and upregulate Bcl6. These cells upregulate activation-induced deaminase (AID) expression and will undergo CSR and somatic hypermutation. Somatic hypermutation induces point mutations in the recombined V(D)J genes. These mutations may lead to increased or decreased affinity for the antigen to which the BCR is specific. Within the follicles, follicular DCs present antigens to B cells, which compete for binding of the antigen. A B cell expressing a BCR with high affinity for the antigen will receive strong survival signals and as a result antigen-specific B cells with a high affinity for the antigen will survive. This process is referred to as affinity maturation.[47, 48] The B cells that emerge from such a germinal center reaction will either become circulating memory B cells or plasma cells. The majority of plasma cells will home to the bone marrow where some of them will find a niche, where they can survive for many years as long-living immunoglobulin-secreting plasma cells that provide a constant source of high-affinity antigen-specific immunoglobulins.[49]

B cell responses to thymus independent antigens

Extrafollicular B cells including B-1 cells and MZ B cells are the main B cell subsets that mount rapid thymus-independent (TI) humoral responses. The term TI originates from experiments performed with mice lacking a thymus and as a result lacking T cells. B cell responses that are not significantly impaired in thymectomized mice are considered to be independent of T cell help. TI responses are rapidly induced mostly in response to conserved microbial carbohydrate or glycolipid structures. These responses result in the production of polyspecific low affinity IgM antibodies and do not involve somatic hypermutation. Extrafollicular B cells receive activation signals that resemble CD40 ligation in the form of secreted factors like B-cell activating factor of the TNF-family (BAFF) and a proliferation-induced ligand (APRIL) that are produced among others by TLR-activated DCs and macrophages.[47]

Immunoglobulins

Immunoglobulins (alternatively named antibodies) are identical to the BCR of the B cell from which they originate, with the exception of a C-terminal sequence that anchors the molecule to the cell membrane. As a result immunoglobulins are secreted and do not form surface-bound receptors. Immunoglobulins are key mediators of humoral immunity that are secreted by plasma cells and are found in blood and tissue fluids of vertebrates. They are composed of two identical pairs of heavy and light chains (illustrated in figure 3). Immunoglobulin light chains consist of a constant (C_L) and a variable (V_L) domain. Immunoglobulin heavy chains consist of a variable domain (V_H) together with 3 constant domains (C_H) and a hinge region (IgG, IgA and IgD) or 4 C_H domains and no hinge region (IgM and IgE). A functional immunoglobulin monomer is composed of two pairs of heavy and light chains that are linked by disulfide bridges between their hinge regions (IgG, IgA and IgD) or between their C_H2 domains (IgM and IgE).

Immunoglobulins have a functionally polarized structure, with on one side the Fab region harboring a hypervariable region, which is responsible for antigen binding, and on the other side a constant Fc region. The structure of the

constant region determines the effector function of the immunoglobulin. Immunoglobulins are typically classified according to the isotype of their heavy chain. In humans there are five major immunoglobulin heavy chain isotypes: IgM, IgD, IgG, IgA and IgE. Each of these isotypes mediates distinct functions through interaction with specific receptors on effector cells and serum factors. This adds another level of regulation to humoral immune responses. Different immunoglobulin isotypes are secreted as monomers or multimers. IgG and IgE molecules are secreted as a monomer while IgA is typically secreted as a dimer and IgM as a pentamer. The process responsible for the generation of different immunoglobulin heavy chain isotypes is called CSR.

Biological functions of immunoglobulins

Immunoglobulins exert a wide range of biological activities. An important function of immunoglobulins in host defense is neutralization of microbes. Pathogen-specific antibodies can neutralize the capacity of viruses or intracellular bacteria to enter host cells and thereby prevent infection and replication of the pathogen.[27] Immunoglobulins can also activate the complement system by recruiting the complement protein C1 to an opsonized microbe. This initiates the so-called classical complement activation cascade, which ultimately leads to elimination of the target microbe through the formation of a membrane attack complex.[50] Another mechanism through which immunoglobulins regulate immune responses is by interaction with Fc receptors that are expressed on effector cells. This triggers specific responses in effector cells upon recognition of a molecule that is specifically recognized by the Fc receptor-bound immunoglobulin. Some examples of Fc receptor-mediated effector functions of different cell types are degranulation of mast cells and basophils and phagocytosis by neutrophils and monocytes.[27]

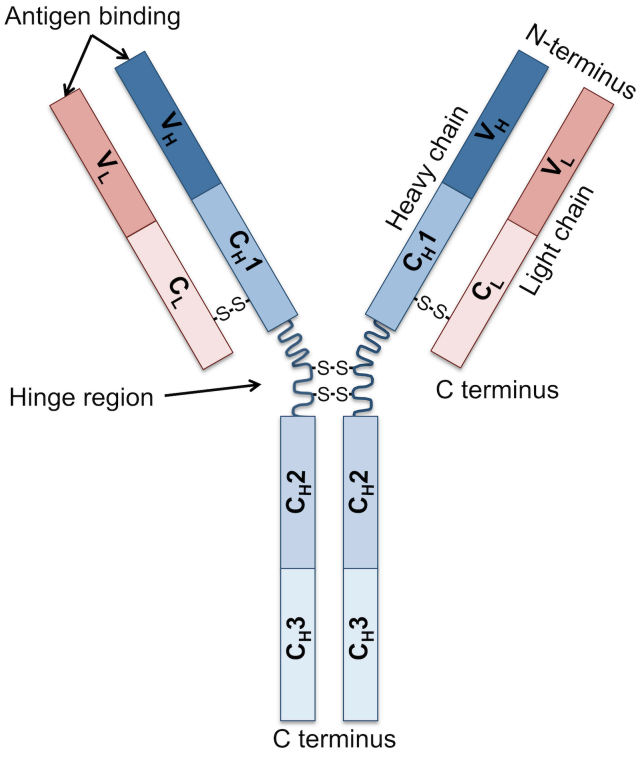

Figure 3. Structure of an immunoglobulin molecule. Immunoglobulins are Y-shaped molecules that consist of two heavy (H) chains and two light (L) chains. The light chains contain a constant (C_L) and a variable (V_L) domain. The heavy chains contain a V_H region and 3 or 4 C_H domains depending on the isotype. IgG, IgA and IgD have 3 C_H domains and a hinge region between C_H1 and C_H2 while IgE and IgM have 4 C_H domains and no hinge regions. The two heavy chains are linked through disulfide bonds (S-S) between their hinge regions (IgG, IgA and IgD) or between their C_H2 domains (IgE and IgM) and each H chain is linked to a L chain by a disulfide bond. Adapted from[27].

Class switch recombination

CSR is the mechanism that is required for the production of different immunoglobulin isotypes. To understand the process of CSR, it is important to understand the structure of the immunoglobulin (Ig) heavy (H) chain locus. Each Ig molecule consists of two heavy (IgH) and two light chains, both of which both contain variable (V) and constant (C) regions. The genes encoding the heavy chains of human immunoglobulins are located on chromosome 14. The region of the heavy chain that determines antigen-specificity is made up by the variable (V_H), diversity (D) and joining (J_H) exons that are randomly combined during early B cell development to form a $V_H(D)J_H$ cassette. The $V_H(D)J_H$ is located upstream of the C exons. The C region of the IgH chain

determines the isotype of the Ig. Each C_H gene has its own promoter, an intervening (I) exon and a switch (S) region directly upstream of the coding exons.[51, 52] In mature naïve B cells, the $V_H(D)J_H$ cassette is linked to the constant region of the μ chain (Cμ).[53] Consequently, mature naïve B cells express surface IgM and, as a result of alternative splicing, IgD as their BCR (Illustrated in figure 4).

Upon activation, naïve B cells can undergo CSR, a process in which the B cell changes the isotype of the produced immunoglobulin while its antigen-specificity is retained. There are 9 human IgH chain isotypes: IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE and 8 murine Ig isotypes: IgM, IgD, IgG1, IgG2a, IgG2b, IgG3, IgA and IgE.[51] Essentially CSR is the process in which double strand breaks are generated at two different S regions within the IgH chain locus followed by the deletion of the intervening DNA and ligation of the remaining S regions. The S regions are located upstream of the gene segments encoding the various IgH chains. The generation of double strand (ds) DNA breaks at selected S regions requires the activity of several enzymes that include AID, uracil DNA glycosylase (UNG) and apyrimidic/apurinic endonucleases (APE).[54] The expression of ε-germline transcripts precedes CSR to IgE and is required for IgE synthesis. The proposed mechanism by which germline expression facilitates CSR is the ‘accessibility model’. This model implies that germline transcription (induced by cytokines) leads to opening of the chromatin structure of the S region making it accessible to AID. AID converts cytosines to uracils in such a transcriptionally active S region.[55-57] These uracils can be excised by the DNA base excision repair enzyme UNG leading to the generation of abasic sites that can be processed by APE1 leading to the formation of nicks and ultimately result in dsDNA breaks.[58] The dsDNA breaks are repaired by the non-homologous end-joining repair system resulting in the ligation of a downstream C_H region (Cε in the case of IgE CSR) next to the VDJ gene segments (Figure 4).[52] The ends of the excised DNA can be joined resulting in the formation of an extrachromosomal circular DNA (also referred to as

excision circle). These excision circles can be detected using polymerase chain reaction (PCR) and are indicative of a recent CSR event.

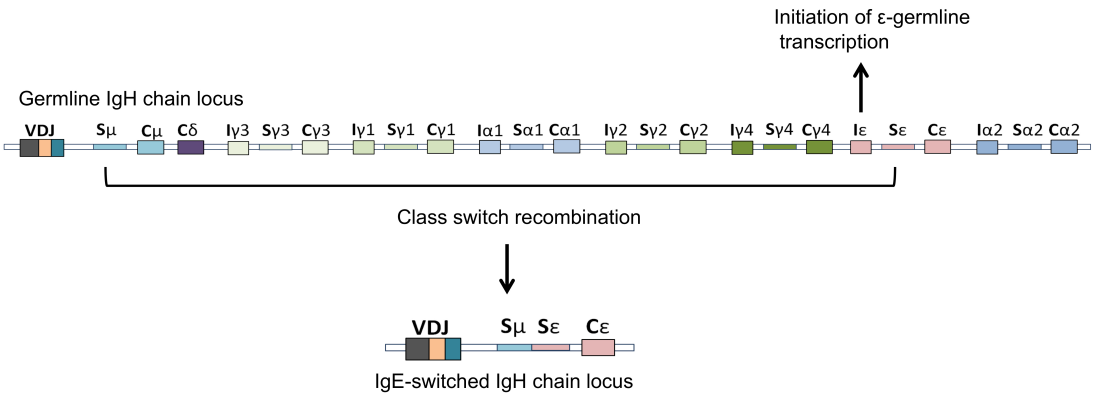

Figure 4. Organization of the human immunoglobulin heavy chain locus in mature naive B cells and class switch recombination to IgE. Mature naive B cells carry the germline IgH chain locus. These cells have their rearranged $V_H(D)J_H$ domain located directly upstream of the Cμ domain and as a result they express IgM and IgD as surface receptors. CSR to IgE is requires ε-germline transcription and finally results in the generation of double strand DNA breaks at Sε followed by ligation of the Sμ to Sε. Adapted from[59].

The selection of the C_H chain gene that will be recombined to VDJ region depends on the expression of germline IgH gene expression. In murine B cells it has been shown that IL-4 selectively induces ε and γ1-germline transcription while IFN-γ selectively induces γ2b germline transcription.[60] In human B cells, there is not such a specific regulation of germline transcripts. It was found that γ1, γ2, γ3 and ε germline transcripts are constitutively expressed in naïve tonsillar B cells. Stimulation with anti-CD40 + IL-4 upregulated expression of γ1, γ2, γ3, γ4 and ε germline transcripts. ε germline transcript expression was however upregulated 10-fold stronger in response to anti-CD40 + IL-4 than γ germline expression.[61] On the other hand, IL-10 enhances survival, proliferation, differentiation and isotype switching of human B cells.[62] IL-10 augments IgG4 production, whereas it inhibits IL-4-induced IgE CSR.[63-65]

Immunoglobulin isotypes

IgM

IgM makes up 5-10% of the total circulating immunoglobulins and is secreted from plasma cells mostly as a pentamer (and to a lesser extent as a hexamer) containing five (or six) IgM molecules that are covalently linked at their Fc regions by a J-chain. Because of its large size, IgM does not easily diffuse and is found in interstitial fluids only in very small amounts. The presence of the J-chain allows active transport of IgM by epithelial cells and, as a result, IgM can be secreted into mucosae. IgM is usually produced early during the course of an infection and mainly functions by opsonizing microbes and targeting them for complement-mediated destruction. Antigen-specific IgM antibodies generally have a lower affinity for their antigens compared to other immunoglobulin isotypes. Despite this, IgM is a potent complement activator, a feature that primarily results from its multimeric structure and its consequent high avidity.

IgD

Circulating IgD can be detected only in small amounts. It has a short serum half-life and constitutes less than 0.5% of the total serum immunoglobulins. In contrast to its secreted form, membrane-bound IgD is abundantly expressed on mature naïve B cells, which co-express IgM. Recently, it was found that a small subset of human B cells expresses IgD in absence of IgM. This has been attributed to antigen-induced transcriptional inactivation of the IgM locus and not to classical CSR. These cells are mainly found in the upper respiratory mucosa.[66] The biological function of secreted IgD remains largely unknown. There have been no specific Fc receptors identified that bind IgD, but IgD can bind to basophils, mast cells, neutrophils and myeloid DCs.[67]

IgG

IgG is the most abundant immunoglobulin isotype in circulation as it accounts for 80% of all serum immunoglobulins. In humans there are four subtypes of IgG (IgG1-4). The numbering of IgG subtypes from one to four corresponds to the concentrations that are normally found in serum. IgG1 is found at the highest concentration, while IgG4 has the lowest concentration. IgG antibodies can cross the placenta and are the primary source of maternal

antibodies that are transferred to the developing fetus. These four IgG subtypes show 95% homology, but have some structural differences that are mainly found in the size of their hinge region and the number and position of interchain disulfide bonds, which link the two heavy chains. The primary functions of IgG antibodies are clearance of pathogens by opsonization leading to complement activation and Fc-receptor-mediated effector cell activation.[68] As a result of their structural differences, the different subtypes of IgG have distinct biological functions, which will be briefly discussed below.

There are five different Fcγ receptors that have different affinities for the individual IgG subclasses. Four of these are activating receptors that carry immunoreceptor tyrosine-based activation motif (ITAM) sequences in their intracellular domains or on their associated subunits. These activating Fcγ receptors are: FcγRI (alternative name CD64), FcγRIIA (alternative name CD32A), FcγRIIIA (alternative name CD16A) and FcγRIIIB (alternative name CD16B). There is one inhibitory Fcγ receptor: FcγRIIB (alternative name CD32B). FcγRIIB carries an immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRI is a high affinity IgG receptor while the other Fcγ receptors have low affinity for IgG.[69] Most immune cells of the hematopoietic system express one or more types of activating Fcγ receptors as well as inhibitory Fcγ receptors. NK cells only express activating receptors while B cells only express inhibitory receptors and T cells do not express any Fcγ receptors. As most immune cells express both activating and inhibitory Fcγ receptors, the effect of Fcγ receptor ligation depends on the interplay between these two types of receptors. Depending on the cell type involved activating Fcγ receptors can mediate degranulation, phagocytosis, cytokine production or antibody-dependent cellular cytotoxicity. Inhibitory FcγRIIB on B cells functions as a sensor for IgG and thereby facilitates a feedback inhibition loop that suppresses B cell activation and can even induce apoptosis of low affinity or autoreactive B cells.[68]

IgG1 is the most abundant human IgG subtype. IgG1 has a 15 amino acid-long hinge region that gives it a relatively high degree of flexibility. IgG1 binds

to all Fcγ receptors with relatively high affinity and as a result it is efficient at mediating microbial clearance through phagocytosis by effector cells.[70] IgG1 responses are primarily mounted against protein antigens. IgG1 can activate the complement system with intermediate efficiency.[71]

IgG2 has a low affinity for all Fcγ receptors and only shows detectable binding to FcγRII.[70] Therefore IgG2 is a relatively weak mediator of microbial clearance through effector cells. It also is a poor activator of the complement system. IgG2 responses are typically mounted against polysaccharide antigens.[71]

IgG3 has a relatively high binding affinity for all Fcγ receptors.[70] It is the strongest complement activating IgG subtype. Like IgG1, IgG3 responses are primarily mounted against protein antigens.[71]

In healthy individuals IgG4 has the lowest serum concentration of all IgG isotypes. IgG4 binds most Fcγ receptors with lower affinity than IgG1 and IgG3 but with higher affinity than IgG2. Interestingly the FcγRIIB binds to IgG4 with a higher affinity than to any other IgG subclass.[70] IgG4 can not activate the classical complement pathway.[72] An interesting feature of IgG4 is its inability to cross-link antigens. This was initially observed using sera from individuals with high levels of allergen-specific IgG4, which showed impaired capacity to cross-link fluid-phase allergen to solid-phase allergen. It was also shown that IgG4 did not precipitate with allergen while IgG1 did.[73] Thus it was concluded that IgG4 was a non-precipitating, functionally monovalent immunoglobulin isotype. This functional monovalency results from the unique capacity of IgG4 antibodies to rearrange their heavy chains by means of a mechanism referred to as Fab arm exchange, which involves the exchange of Fab arms between two different IgG4 molecules by swapping a heavy chain and attached light chain. Support for this model came from the finding that IgG4 antibodies in sera from patients with IgG4 antibodies specific for both house dust mite and grass pollen could induce cross-linking of Sepharose-bound grass pollen antigen to radiolabelled house dust mite allergen *Der p I*.[74]

Furthermore, injection of two monoclonal IgG4 antibodies with different specificities into immunodeficient mice led to the generation of bispecific antibodies while injection of control IgG1 antibodies did not yield bispecific antibodies.[75] This process did not occur readily *in vitro* as it requires the presence of a reducing agent such as reduced glutathionine. Mutagenesis studies demonstrated that the hinge region and the CH3 domain of IgG4 are essential for Fab arm exchange to occur.[75] This process leads to the generation of functionally monovalent antibodies that are bispecific because they contain the variable regions from two different IgG4 antibodies. This feature strongly impairs the ability of IgG4 antibodies to form immune complexes and may result in IgG4-mediated inhibition of immune complex formation by other isotypes.[75, 76] Because of its relatively low affinity for activating Fcγ receptors, its inability to fix complement and its functional monovalency, IgG4 can be regarded as a non-inflammatory antibody isotype. IgG4 production is strongly induced in response to prolonged exposure to soluble protein antigens. Allergen-specific IgG4 antibodies can also compete with IgE for the same antigens and thereby it may dampen IgE-mediated release of inflammatory mediators from basophils and mast cells. Furthermore, allergen-specific IgG4 can inhibit IgE-facilitated antigen-presentation (this process will be discussed below).[77]

IgA

IgA accounts for 10%-15% of the total immunoglobulins present in the body. It exists in serum, but it also is the major immunoglobulin isotype present at mucosal surfaces and in secretions such as saliva and breast milk. Serum IgA exists mostly as a monomer, while IgA that is found at mucosal areas and secretions (frequently referred to as secretory IgA or sIgA) is present as a dimer. Like pentameric IgM, dimeric IgA is formed by linkage of the CH3 domains by a J-chain. This J-chain is required for active transport of the molecule through epithelial cells. In humans, there are two subtypes of IgA; IgA1 and IgA2. Like IgG subtypes, these molecules mainly differ in the structure of their hinge regions. IgA1 has a longer hinge region than IgA2, which makes it more sensitive to bacterial protease digestion and could

explain the relative predominance of IgA2 in mucosal areas while the dominant IgA subtype in serum is IgA1. IgA is important in the protection of mucosal surfaces against pathogens and toxins. It mainly functions through neutralization of these pathogens. IgA is a poor activator of the complement system, but it can bind to Fcα receptors on neutrophils and thereby mediate pathogen clearance through antibody-dependent cell-mediated cytotoxicity. Glycan structures on IgA molecules have been proposed to bind distinct bacteria in a Fab-independent manner. This enables IgA antibodies of different specificities to neutralize common pathogenic bacteria without the need for antigen-specific immunoglobulins.[71]

IgE

IgE has a short serum half-life and accounts for less than 0.01% of the total immunoglobulin in healthy individuals. It is the key immunoglobulin associated with type I hypersensitivity reactions and has been linked with protection against parasitic worm infections.[78, 79] Helminth infections, which are typically chronic, induce strong Th2 responses and induce IgE production. Interestingly the incidence of allergic disorders is lower among people that are infected by helminthes than among non-infected individuals.[80] IgE does not efficiently activate complement.[81] There are two types of Fc receptors that bind IgE; FcεRI and FcεRII (alternatively named CD23). Furthermore a protein called galectin-3 can bind IgE as well as FcεRI.[78] FcεRI is the high-affinity IgE receptor, which is expressed on mast cells, basophils and certain antigen-presenting cells (APCs) (primarily Langerhans cells). FcεRII is the low affinity IgE receptor of which there are two forms; CD23a and CD23b. CD23a is expressed on follicular B cells while CD23b can be induced by IL-4 on T-cells, monocytes, Langerhans cells, eosinophils, macrophages and epithelial cells.[78] The effector mechanisms of IgE and its role in allergic disease are discussed in more detail in the following section.

5.3 Allergic disease

The immunological mechanisms that lead to the development of allergic disease have been extensively studied in the past decades and can be divided into two phases: The sensitization phase and the effector phase, which can be further subdivided into immediate and late responses.[82]

5.3.1 Allergic Sensitization

Allergic sensitization is characterized by the development of allergen-specific T and B cell responses. The initial step in the initiation of allergic sensitization is the priming of allergen-specific naïve $CD4^+$ T cells. Tissue resident DCs capture allergen, mature and migrate to lymph nodes, where allergen-derived antigens are presented to T cells. If these DCs produce Th2-priming cytokines (IL-4 or IL-13), allergen-specific naïve $CD4^+$ T cells differentiate to Th2 cells, which produce IL-4, IL-5, IL-9 and IL-13.[83] As a result of clonal expansion, allergen-specific Th2 cells dramatically increase in number. Furthermore, predominance of Th2 cells in atopic individuals may result from a higher rate of activation-induced cell death of IFN-γ-producing Th1 cells.[84] Th2 derived cytokines have various effector functions including induction of IgE CSR and clonal expansion of allergen-specific B cells, development and recruitment of eosinophils, mucus production, smooth muscle cell contraction and Th2 homing.[85] During the sensitization phase, allergen-specific IgE antibodies are produced as a result of IgE CSR in B cells (illustrated in figure 5). These IgE antibodies circulate and bind to high affinity FcεRI receptors on the surface of mast cells and basophils as well as APCs such as Langerhans cells. This is the key event of sensitization. Subsequent encounter with the same allergen leads to cross-linking of IgE bound to FcεRI on these effector cells and the release of inflammatory mediators and marks the onset of the immediate phase (type 1) hypersensitivity reaction. The mechanisms involved in this process are discussed in more detail below.

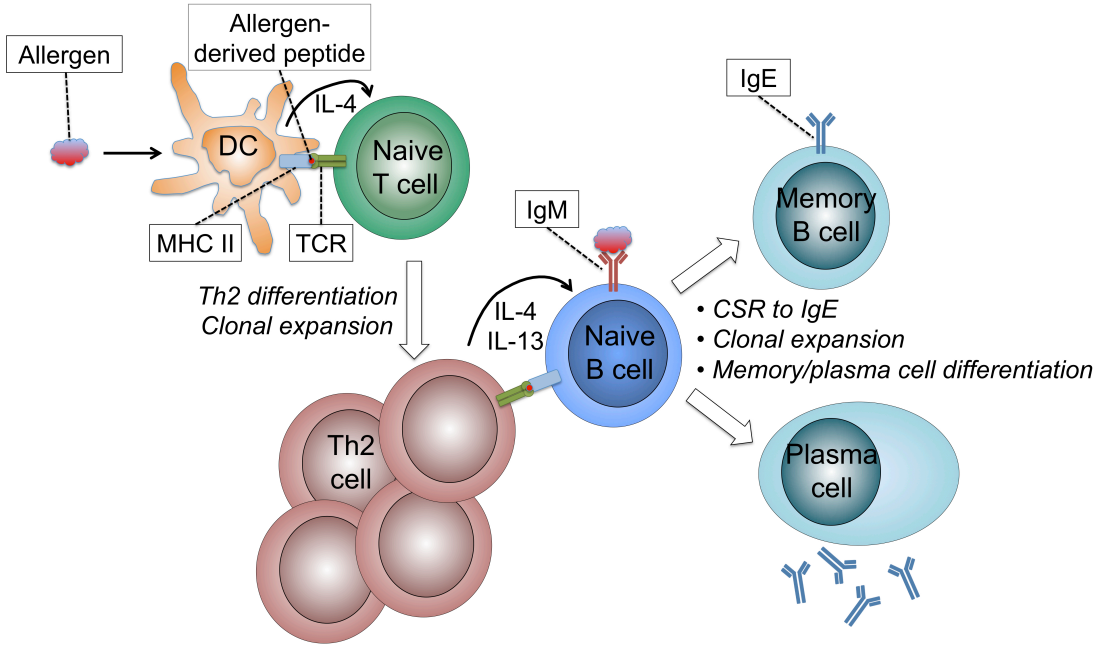

Figure 5. Key events in the process of sensitization to allergens. Upon allergen encounter, DCs capture allergens and migrate to draining lymph nodes where they present allergen-derived peptides to allergen-specific naïve $CD4^+$ T cells. In response to polarizing cytokines produced by DCs, naïve T cells differentiate to Th2 cells and undergo clonal expansion. Allergen-specific naïve B cells bind and internalize allergen and present allergen-derived peptides to Th2 cells. Th2 cells provide IL-4 and IL-13, which facilitate CSR to IgE. Naïve B cells undergo CSR to IgE and differentiate to IgE-switched B cells or IgE-secreting plasma cells. Adapted from[86].

5.3.2 Effector functions of IgE-receptor-expressing cells

The human FcεRI can be expressed as a tetrameric or a trimeric receptor whereas murine FcεRI is only expressed as a tetramer. The tetrameric receptor is expressed by mast cells and basophils and is composed of three different membrane-bound chains: the α, β and γ chains.[87] FcεRI expression enables cells to stably interact with IgE molecules through high affinity binding of the Fc portion of these antibodies to the α-chain of FcεRI, forcing the antigen-specific regions of the IgE molecules to stick out and function as sentinels for their specific antigens. When multivalent allergens bind to cell surface-bound IgE molecules, FcεRI is crosslinked and, dependent on the cell type, cells either release a distinct set of preformed pro-inflammatory mediators (degranulation) or they internalize the captured allergens for subsequent antigen presentation. The early events following antigen-mediated crosslinking of IgE bound to FcεRI are activation of proto-oncogene

tyrosine-protein kinase Src-family kinases followed by tyrosine phosphorylation of cytoplasmic FcεRIβ- and γ-associated ITAMs. These phosphorylated ITAMs provide docking sites for SH2 domains of tyrosine-protein kinase Lyn (LYN), ZAP70 and spleen tyrosine kinase (SYK) which will activate the linker for activation of T cells (LAT) protein which is central to many of the downstream signaling cascades that are required for the release of pro-inflammatory mediators (reviewed in more detail in[88, 89]). Degranulation upon allergen binding occurs in sensitized mast cells and basophils, leading to the release of preformed mediators such as histamine and heparin, and the rapid synthesis of leukotrienes, prostaglandins, cytokines and chemokines. This reaction is referred to as the immediate hypersensitivity reaction. Crosslinking of IgE bound to FcεRI receptors on APCs such as Langerhans cells and DCs can result in internalization and processing allergens. This leads to presentation of allergen fragments on MHC class II molecules together with co-stimulatory molecules, enabling activation of allergen specific CD4+ T cells. Compared to non-sensitized APCs, sensitized DCs or Langerhans cells demonstrate an increased affinity for specific allergens, which enhances the efficiency of allergen internalization. The allergen concentration required for activation of allergen specific T cells is significantly reduced when APC carry allergen specific IgE molecules on FcεRI.[90]

CD23 is the low affinity IgE receptor. The extracellular portion of CD23 contains a C-type lectin domain, which facilitates IgE binding and an α-helical coiled coil stalk that drives the formation of trimers in the cell membrane.[91] Because of the relatively low binding affinity, stable binding of IgE to this receptor occurs mostly when multiple IgE molecules are clustered in complexes.[92, 93] CD23 can be cleaved from the cell surface by ADAM10 and Der p1 (a house dust mite derived protease and a major allergen) yielding soluble CD23 proteins of different sizes.[94, 95]

Besides IgE, a number of other ligands for CD23 have been described. These include CD21 and αMβ2-integrin, αXβ2-integrin, αVβ3-integrin and αVβ5-integrin[78]. Soluble CD23 can have a pro-inflammatory effect as it binds to the

αMβ2-integrin and αXβ2-integrin on monocytes, which respond with nitric oxide production, *cyclic* adenosine monophosphate synthesis and the production of pro-inflammatory cytokines like TNF, IL-1 and IL-6.[96] CD23 and CD21 have been proposed to coordinately regulate IgE production. The binding surfaces on CD23 for IgE and CD21 are spatially separated, which means that CD21 and IgE can bind simultaneously to CD23.[97]. CD23 can interact with CD21 in soluble as well as membrane-bound form. CD23 has been proposed to regulate IgE production both positively and negatively. Soluble CD23 may positively regulate IgE production through simultaneously binding CD21 and membrane IgE on IgE-switched memory B cells. This will culminate in the formation of an extensive signaling platform that might promote the survival and differentiation of IgE-switched B cells. Negative regulation of IgE production can occur by co-ligation of membrane-bound CD23 and IgE by allergen-IgE complexes.[78]

CD23 has been demonstrated to increase the antigen-presenting capacity of B cells through a mechanism referred to as IgE-facilitated antigen presentation (IgE-FAP). B cells are able to internalize, process allergens and present allergenic peptide fragments on MHC class II molecules. This results in the activation of allergen specific CD4+ T cells. B cells expressing CD23 on their surface membrane can bind immune complexes consisting of IgE antibodies and allergens in a B cell receptor-independent manner. This results in the cross-linking of CD23 molecules and subsequent internalization of IC by means of receptor-mediated endocytosis. This mechanism of antigen capture is much more efficient than the uptake and presentation of allergens via pinocytosis and results in the activation of CD4+ T cells at a 100- to 1000 fold lower allergen concentration.[98] Therefore, IgE-FAP could be a major contributor to the induction of CD4+ T cell responses after exposure to low allergen doses. The relevance of this mechanism has been demonstrated for several respiratory allergies such as birch pollen or house dust mite allergy.[98] The first study showing the mechanism of IgE-FAP was published in 1989. This study demonstrated that trinitrophenyl (TNP)-IgE complexes could bind to B cells via CD23 resulting in internalization and activation of TNP-specific T

cells. [99] It has been shown that IgE-allergen complexes can induce up to 10 fold stronger T cell responses *in vivo* than uncomplexed allergen.[100] The first human studies comprised incubation of purified Der p II with sera from atopic patients containing Der p II specific IgE. Subsequently, Epstein–Barr virus (EBV)-transformed B cells were incubated with these IC and used as APC in a lymphocyte proliferation assay. This resulted in strong proliferation of Der p II specific T cells at a 1000-fold lower allergen concentration than required for T cell activation by uncomplexed Der p II. These enhanced antigen-presenting properties are abolished by treatment with anti-CD23 or anti-IgE, stressing that antigen focusing is largely dependent on CD23-IgE interaction. No other APC than B cells show significant surface expression of CD23a on their surface membrane. Interestingly IgE-FAP circumvents the need for an antigen-specific B cell in order to efficiently capture antigens. Therefore it has been proposed that this mechanism could be an important contributor to epitope spreading.[78]

5.3.3 Late phase reactions

Late phase reactions are characterized by the infiltration of Th2 cells and eosinophils to the site of allergen exposure. Cytokines produced by activated Th2 cells include IL-4, IL-5, IL-9 and IL-13. These factors mediate IgE induction, eosinophil infiltration, mucus production and smooth muscle cell contraction. If the allergen persists the late phase response can convert into a chronic inflammatory response, which can manifest for example as chronic asthma or atopic dermatitis.[86]

5.4 Healthy immune responses to allergens

Allergic responses are the result of an imbalanced response to innocuous environmental antigens that leads to the production of Th2 cells and IgE antibodies. Some individuals develop allergies while others do not and the mechanisms by which immune responses to innocuous antigens lead to either allergy or non-harmful immunity remain largely unknown. One possibility is that healthy individuals simply do not mount an immune response to allergens. This is certainly not always the case since allergen-specific

immunoglobulins and T cells can be frequently found in healthy individuals.[101] Allergen-specific Th cells play a key role in the initiation of allergic responses. The frequencies of allergen-specific Th1, Th2 and Tr1 can be determined by labeling cytokine-producing $CD4^+$ T cells in response to stimulation with environmental allergens. IFN-γ- (Th1 cells), IL-4- (Th2 cells) and IL-10- (Tr1 cells) producing Th cells show different frequencies in allergic individuals compared to healthy individuals. IL-10-producing Tr1 cells are the dominant allergen-specific Th cell subset while IL-4-producing Th2 cells have a high frequency in allergic individuals.[101] As mentioned before, Tr1 cells use different mechanisms to suppress immune responses including production of IL-10 and TGF-β and surface expression of CTLA-4 and PD-1. Despite differences in the frequencies of Th cell subsets, both healthy and allergic donors have allergen-specific Th1, Th2 and Tr1 cells. This suggests that changes in the frequencies of these subsets may result in development of allergy or recovery.[101]

Healthy immune responses to high-dose allergen exposure in humans have been studied in two different models: Immune responses to bee venom allergens in beekeepers and immune responses to cat allergens in cat owners. The beekeeper model is an elegant model that can be used to study the mechanisms of immune tolerance to venom antigens. During the season, beekeepers receive multiple bee stings and as a result these individuals are repeatedly exposed to high dose bee venom antigen. After several bee stings, Th1 and Th2 cells specific for the major bee venom allergen Api m 1 show a switch towards Tr1 cells. At the same time cutaneous late-phase swelling responses are suppressed. These changes persist as long as exposure to bee venom is present and return to initial levels several months after the end of the beekeeping season.[102] Furthermore, bee venom allergen-specific IgG4 levels are high in beekeepers and the ratio of specific IgG4/IgE is approximately 1000 times higher than in allergic individuals.[103] Similar findings were observed upon exposure to cat allergens, which induced cat allergen-specific IgG4 antibodies and IL-10-producing Tr1 cells.[104, 105]

5.5 Allergen-specific immunotherapy and peripheral tolerance

Allergen-specific immunotherapy (SIT) is the only treatment that can induce to long-term improvement of clinical allergy symptoms. The principle of allergen-SIT has already been applied for over a century and involves repeated administration of allergens to an allergic patient. The earliest reports on the protective effects of allergen-SIT against allergies date back to the beginning of the 20th century when Noon and Freeman were the first to experiment with allergen-SIT. They reported clinical improvement in hay fever patients after applying subcutaneous injections of grass pollen extracts.[106, 107] The basic principle of allergen-SIT has not changed much since then. This principle comprises repeated administration of increasing doses of allergen. Allergen-SIT is most frequently applied for the treatment of hymenoptera venom allergies and allergic rhinitis caused by grass or tree pollen as well as house dust mite.[108]

Many patients that receive allergen-SIT become tolerant to late-phase skin responses at early stages of the therapy. One of the events associated with this observation is that mast cell and basophil degranulation activity is reduced already after the first administration of allergen. The mechanism underlying this effect remains largely unknown. A role for histamine receptor (HR) 2 expression on basophils in the suppression of basophil degranulation has been suggested. A rapid upregulation of HR2 expression on basophils was observed in allergic patients receiving SIT. HR2 expression was strongly upregulated within 6 hours after the initiation of SIT. HR2 strongly suppressed FcεRI-mediated basophil degranulation.[109] Furthermore a switch from a Th2 cytokine profile to a Th1 cytokine profile was observed in response to allergen-SIT as was measured by stimulating PBMC with relevant allergens.[110, 111]

Induction of a tolerant state in peripheral T cells is another critical step for the success of allergen-SIT. Both inducible Tr1 cells and thymic-selected $CD4^+CD25^+$ Treg cells play a role in the induction of a tolerant state in

peripheral T cells. This tolerant state is characterized mainly by the induction of antigen-specific Tr1 cells, which produce IL-10 and TGF-β.[112, 113] Furthermore, $CD4^+CD25^+$ Treg cells from atopic donors could only poorly suppress proliferation of effector T cells.[114]

Humoral immune responses are also affected by allergen-SIT. Allergen-specific IgE levels transiently increase during the first months of allergen-SIT but decline in the later course of allergen-SIT. Circulating allergen-specific IgG antibodies show a gradual increase starting from the early phases of allergen-SIT. Allergen-specific IgG4 levels show the strongest increase during the course of allergen-SIT. This can result in decreases in the ratio of allergen-specific IgE:IgG4 of 10- to 100-fold.[115, 116] IgG antibodies can be directed against the same epitopes as IgE and therefore could function as blocking antibodies that compete with IgE for the same epitope. There have been mixed reports on the correlation between increases in allergen-specific IgG levels and clinical outcome of allergen-SIT.[117, 118] It can be argued that rather than the absolute concentration of allergen-specific IgG antibodies, one should assess the affinity and/or blocking activity of allergen-specific IgG to find a good correlation with clinical outcome of allergen-SIT.

The decreased ratio of allergen-specific IgE/IgG4 that is frequently observed during the course of allergen-SIT can be attributed to some extent to a skewing from allergen-specific Th2 cells towards Treg cell predominance. These cells could play a role in regulating both IgE and IgG4 production. Both IgG4 and IgE are induced upon stimulation with IL-4 and CD40L. The addition of IL-10 however has opposite effects on IgE and IgG4 production. IgE production is suppressed by IL-10 while IgG4 production is augmented in the presence of IL-10.[64] Furthermore it was shown that co-culturing IL-4 + CD40L-stimulated PBMC as well as B cells with IL-10-secreting Tr1 cells or $CD4^+CD25^+$ Treg cells results in reduced IgE production and increased IgG4 production. In contrast, addition of $CD4^+CD25^-$ effector T cells to IL-4 + CD40L-stimulated PBMC or B cells did not affect IgE or IgG4 production.[65] These findings indicate that IL-10, Treg and Tr1 cells regulate antibody

isotype formation and skew the specific response from an IgE- to an IgG4-dominated phenotype.

5.6 Cytokines associated with peripheral tolerance

IL-10 and TGF-β are the cytokines involved in the immunosuppressive function of inducible regulatory T cell subsets. IL-10 is secreted as a homodimer and binds to a tetrameric receptor complex that is composed of two IL-10R1 and two IL-10R2 chains.[119, 120] The IL-10R1 chain is expressed on many cell types including B and T cells, NK cells, monocytes, mast cells and DCs while the IL-10R2 chain is ubiquitously expressed.[121] IL-10 receptor signaling is mediated by activation of Jak1 and Tyk2 followed by phosphorylation of signal transducer and activator of transcription (STAT) 1, STAT3 and STAT5.[122] Human IL-10 is primarily produced by monocytes, T cell subsets, DCs, macrophages, B cell subsets and mast cells.[121, 123] The mechanisms regulating expression of the *IL-10* gene have not been fully elucidated but expression of IL-10 requires the ubiquitously expressed transcription factors Sp1 and Sp3.[124] Furthermore the 3' untranslated region of the IL-10 mRNA contains multiple copies of mRNA destabilizing motifs indicating that IL-10 production is also regulated by posttranscriptional mechanisms.[125] IL-27 potently induces IL-10 expression in T cells in a STAT1- and STAT3-dependent manner.[126] In addition IL-27 can promote Tr1 differentiation from naïve cells by induction of c-Maf and AhR expression.[127]

As mentioned earlier, IL-10 is a key immunosuppressive cytokine that plays an important role in the protection of the host against exaggerated inflammatory responses to infections as well as chronic inflammatory diseases such as autoimmunity and allergy. IL-10 directly suppresses the antigen-presenting capacity of APCs by inducing downregulation of the co-stimulatory molecules CD80 and CD86.[62] Furthermore, IL-10 inhibits the production of a large number of cytokines and chemokines[62] and suppresses $CD4^+$ T cell proliferation mainly indirectly through its suppressive effects on APCs. Interestingly, IL-10 can also directly suppress T cell cytokine production through suppression of CD28 and ICOS.[128] In contrast to its

suppressive effects on a variety of cells, IL-10 enhances survival, differentiation and CSR of both human and murine B cells.[129-131]

TGF-β is a pleiotropic cytokine that is produced by many different cell types of both hematopoietic and non-hematopoietic origin. It plays a role in a wide variety of biological processes including embryonic development, wound healing and immune responses. In mammals three isoforms of TGF-β are expressed: TGF-β1, TGF-β2 and TGF-β3.[132] TGF-β1 is the main TGF-β isoform expressed in immune cells. TGF-β is secreted in a latent form complexed with latent TGF-β binding protein and latency-associated peptide. This complex can be activated through proteolytic cleavage by proteases such as plasmin or matrix metalloproteases.[132] The first evidence for an immunoregulatory function of TGF-β came from the generation of mice with a targeted mutation of the TGF-beta 1 allele. These animals showed no developmental abnormalities, but developed a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure and death.[133] TGF-β is a key cytokine produced by human and murine $CD4^+CD25^+$ nTregs as well as inducible Th1 and Th3 cells.[40]

5.7 Regulatory B cells

5.7.1 Cytokine production by B cells

The classical function of B cells in immune responses is the production of antibodies. It was only recently that their capacity to produce cytokines was recognized as a relevant feature of this cell type. Much like Th cells, B cell subsets can be categorized based on the cytokines that they produce. Studies using mouse models have demonstrated functional B effector (Be) 1 and Be2 subsets. INF-γ- and IL-12-producing Be1 cells and IL-4- and IL-6-producing Be2 cells were obtained upon co-culturing naïve B cells with polarized Th1 or Th2 cells respectively. Under these conditions, both Be1 and Be2 cells produced IL-10. Interestingly, this polarizing effect was reciprocal, as *in vitro* differentiated Be1 cells could drive naïve $CD4^+$ T cells to Th1 cells while *in*

vitro differentiated Be2 cells could drive naïve $CD4^+$ T cells to Th2 cells.[134] So far, clear Be1 and Be2 subsets have not been identified in humans.

5.7.2 Regulatory B cells in murine autoimmune models

The earliest studies that indicated the existence of B cells with suppressive capacity date back to 1974. Adoptive transfer of B cell-depleted splenocytes did not inhibit delayed-type hypersensitivity (DTH) skin reactions in guinea pigs.[135, 136] Over 20 years later a study using B cell-deficient (μMT) mice demonstrated that, unlike wild-type (wt) mice, μMT mice did not recover from EAE. This finding suggested that B cells might be able to protect against autoimmune disease. A key role for IL-10-producing B cells in protection against EAE was demonstrated using chimeric mice that specifically lacked IL-10 expression in B cells. Like μMT mice, these mice did not recover from EAE. Similarly, chimeric mice that specifically lacked CD40 expression in B cells failed to recover from EAE. These findings indicate that B cells can regulate autoimmunity by provision of IL-10 which requires a CD40-mediated signal.[137]

At the same time another group found that under chronic intestinal inflammatory conditions a B cell subset is induced, which is characterized by upregulation of CD1d and IL-10 production. This B cell subset could suppress the progression of intestinal inflammation by downregulation of IL-1-associated inflammatory cascades and STAT3 activation.[138] Several years later, the same group reported that $CD1d^{hi}CD5^+$ B cells could negatively regulate T cell-mediated inflammatory responses as demonstrated in an oxazolone-induced contact hypersensitivity (CHS) model. IL-10 production was induced upon LPS stimulation (and to a lesser extent by anti-IgM + anti-CD40 mAb) and was largely restricted to this B cell population, which they designated 'B10' cells. $CD1d^{hi}CD5^+$ B cells make up 1%-2% of spleen $B220^+$ cells and 7%-8% of peritoneal cavity B cells in wt mice but were absent from blood or lymph nodes. Adoptive transfer of wt $CD1d^{hi}CD5^+$ $B220^+$ splenocytes isolated from oxazolone-sensitized mice into oxazolone-sensitized $Cd19^{-/-}$ mice (which lack the BCR co-receptor CD19 and have reduced numbers of

B10 cells) significantly reduced ear swelling upon challenge. This protective effect was not transferred by non-CD1d^{hi}CD5^{+} B220^{+} wt splenocytes, neither by CD1d^{hi}CD5^{+} B220^{+} splenocytes isolated from sensitized IL-10$^{-/-}$ mice. This indicates that protection against T cell-mediated inflammation in a CHS model can be mediated by CD1d^{hi}CD5^{+} B cells and is dependent on IL-10.[139] Recently it was shown that maturation of functional B10 cells that can suppress EAE requires IL-21 and CD40-dependent interaction with T cells. Furthermore *in vitro* stimulation of splenic B cells for four days with CD40L, BAFF and IL-4 followed by five days stimulation with IL-21 drives B10 cell development and induces strong proliferation of B10 cells. Adoptive transfer of *in vitro* expanded B10 cells could inhibit established EAE in recipient mice.[140]

To determine the role of TLR-activated B cells on EAE, several B cell-restricted TLR and MyD88 deficient mice were studied. Splenic B cells from C57BL/6 mice were shown to secrete IL-10 in response to LPS and CpG, while stimulation with anti-CD40 and/or anti-Igκ mAbs did not induce IL-10. This observation does raise questions about the different stimuli that are used by different research groups to induce IL-10 production in B cells, since several other studies described significant IL-10 production upon stimulation with anti-CD40 mAb and/or BCR crosslinking reagents.[137, 139, 141] As expected, LPS-induced IL-10 production was abrogated in splenic B cells from TLR2/4$^{-/-}$ as well as MyD88$^{-/-}$ mice while CpG-induced IL-10 production was only abrogated in splenic B cells form MyD88$^{-/-}$ mice. Chimeric mice lacking MyD88 only in B cells did not recover from EAE induced by MOG-induced EAE. These mice displayed a stronger self-reactive T cell proliferative response in combination with elevated IL-17 and IFN-γ production compared to wt mice.[142]

As was shown in another study, chimeric mice lacking MyD88 only in B cells showed improved control of bacterial replication and prolonged survival upon *Salmonella typhimurium* infection compared to wt mice. MyD88 signaling in B cells suppressed neutrophils, NK cells and inflammatory T cells leading to

impaired clearance of *Salmonella typhimurium.* The key mediator in this process was IL-10.[143]

Infection of C57BL/6 mice with *Helicobacter felis*, a strain that is closely related to the human gastrointestinal pathogen *Helicobacter pylori,* leads to development of early gastric carcinogenesis in a subset of mice, characterized by infiltration of inflammatory cells, atrophic gastritis, hyperplasia and intestinal metaplasia. Th1-polarized cells are the main effector cells that drive this pathology while at the same time they are required for control of the infection. A key role for IL-10-producing B cells in the prevention of formation of gastric premalignant lesions was demonstrated. B cells can be activated by *Helicobacter*-derived TLR-2 ligands in a MyD88-dependent manner leading to IL-10 production and IL-10-dependent suppression of $CD4^+$ T cells and the induction of Tr-1 cells. This suppression depended on TCR signaling, CD40/CD40L and CD80/CD28 interaction.[144]

Stimulation of splenocytes isolated from arthritic mice with agonistic anti-CD40 mAb induced IL-10-producing B cells. When adoptively transferred into DBA/1-TcR-β-Tg mice (transgenic mice expressing the collagen type-II-specific β-chain, which develop a severe form of chronic relapsing polyarthritis that shares characteristics with human rheumatoid arthritis[145]), these cells could inhibit Th1 differentiation and arthritis in a collagen-induced arthritis (CIA) model.[146] To address the relative role of B cells at different stages of development in the protection against CIA, different types of transitional and mature B cells were transferred during the induction phase of CIA. Only the transfer of transitional 2 marginal zone precursor (T2-MZP) cells (identified as $AA4^+CD21^{high}$ $CD23^+CD24^{high}IgM^{high}IgD^+CD1d^+$) could inhibit Th1 responses, DTH responses and the development of arthritis by provision of IL-10.[147]

CD40 ligation with anti-CD40 mAb was shown to enrich Bregs upon short-term *in vitro* culture. Transfer of *in vitro* anti-CD40-generated T2 B cells derived from mice with established lupus could significantly improve renal disease and survival. These cells suppressed Th1 responses and induced

differentiation of IL-10-producing Tr1 cells. Furthermore, *in vivo* application of agonistic anti-CD40 could reverse established lupus.[141]

Administration of apoptotic cells together with OVA peptide and complete Freund's adjuvant into mice carrying OVA-specific transgenic T cells (DO11.10) induced a dramatic increase in OVA-specific IL-10 secretion. The initial increase in IL-10 production was derived from splenic B cells that responded directly to apoptotic cells and in turn induced IL-10 in antigen-specific CD4+ T cells. This was accompanied by a reduction of circulating pathogenic anti-collagen II antibodies. Transfer of B cells from apoptotic cell-treated mice conferred protection to CIA.[148]

Chimeric mice lacking IL-10 specifically in B cells (IL-$10^{-/-}$ B cell) have been used to study the role of B cell-derived IL-10 in several models of chronic inflammation. These mice develop more severe arthritis compared to wt B cell mice in a methylated BSA arthritis model. This was accompanied by an increase in Th1 and Th17 cells and a decrease in the number of $Foxp3^{+}$ regulatory T (Treg) cells, which expressed lower levels of Foxp3. Transfer of wt T2-MZP B cells but not follicular of marginal zone B cells to arthritic IL-$10^{-/-}$ mice could restore the frequencies of Th1, Th17 and Treg cells to those observed in wt mice, demonstrating that this subset of B cells is able to confer protection to methylated bovine serum albumin (BSA)-induced arthritis.[149]

$TCR\alpha^{-/-}$ mice develop spontaneous chronic colitis, which closely resembles human ulcerative colitis. This pathology is mediated by autoreactive $CD4^{+}$ $TCR\alpha^{-}\beta^{low}$ T cells and is characterized by the presence of autoantibodies. The disease was further exacerbated $TCR\alpha^{-/-}$ crossed with $Ig\mu^{-/-}$ (B cell deficient) mice. Adoptive transfer of B cells could suppress pathogenic $CD4^{+}$ $TCR\alpha^{-}\beta^{low}$ T cell responses and colitis. B cells derived from CD40-deficient mice or wt B cells that were pretreated with a blocking anti-CD40 mAb were unable to prevent disease symptoms.[150]

5.7.3 Regulatory B cells in allergy

A role for IL-10-producing B cells in the protection against allergic responses has been demonstrated in several mouse models.

To study the role of B cells in the modulation of allergic airway disease an OVA model was used to induce allergic airway inflammation or local inhalational tolerance. Allergic airway inflammation manifests when OVA-sensitized mice are exposed to aerosolized OVA for a short period (1 hour per day for 7 days) while local inhalational tolerance occurs upon chronic exposure (daily challenge for 42 days) to OVA. Local inhalational tolerance was characterized by minimal airway and tissue eosinophilia, reduced airway lymphocytosis, absence of airway hyper-reactivity but persistent high levels of OVA-specific IgE and IgG1. $JhD^{-/-}$ mice (mice that have a targeted mutation in their J_H locus, which prevents rearrangement of the V(D)J recombination and blocks B cell development, rendering these mice B cell deficient[151]) developed local inhalational tolerance normally. However adoptive transfer of B cells isolated from hilar lymph nodes of chronically OVA-exposed mice to OVA-sensitized recipients could suppress allergic airway inflammation. B cells isolated from short-term challenged mice did not have such a suppressive effect. These findings support a role for B cell in mediating tolerance. *In vitro* experiments demonstrated that B cells isolated from hilar lymph nodes of chronically OVA-exposed mice mediated TGF-beta-dependent conversion of T effector cells into functionally suppressive $CD4^+CD25^+Foxp3^+$ Treg cells. Interestingly, it seems that B-cell-mediated suppression of allergic airway inflammation did not require IL-10 since local inhalational tolerance was maintained in IL-10-deficient mice. Furthermore, blocking of IL-10 did not inhibit B cell-mediated *in vitro* Treg cell conversion.[152]

Chronic helminth infections have frequently been associated with reduced incidence of atopy.[153] Regulatory B cells may be involved in protect against allergic inflammation as a result of chronic helminth infections. This has been demonstrated by several studies.

Mice infected with *Schistosoma mansoni* were protected from anaphylaxis in a Pen V model. Worm-infected mice did not show a decrease in body temperature after being passively sensitized and challenged with antigen. This protective effect of *Schistosoma mansoni* infection on systemic anaphylaxis was dependent on IL-10-producing B cells. The frequency of IL-10-producing B cells was two-fold higher in *Schistosoma mansoni*-infected mice compared to uninfected mice. B-1-deficient mice were still resistant to anaphylaxis in this model indicating that a B-2 cell population mediates resistance from anaphylaxis. Interestingly, adoptive transfer of B cells from worm-infected wt mice did not protect from anaphylaxis. Only B cells from IL-$4^{-/-}$ mice conferred protection to anaphylaxis in recipient mice.[154]

A role of helminth-induced regulatory B cell responses in the protection against allergic airway responses was demonstrated by infecting mice with the helminth *Schistosoma mansoni*. These mice were subsequently sensitized and challenged with OVA to induce airway inflammation. In order to determine the effect of acute and chronic helminth infection, mice were sensitized and challenged at 8 (acute infection), 12 (intermediate infection) or 16 weeks (chronic infection) after helminth infection. Allergic airway inflammation was more severe during acute helminth infection but ameliorated during chronic infection. OVA-specific IgE levels were not affected by helminth infections. Mesenteric lymph node cells or splenocytes from chronically helminth-infected mice secreted less IL-13, IL-4 and IFN-γ upon stimulation with OVA or helminth antigens than cells isolated from uninfected mice. IL-10 production in response to restimulation with OVA or helminth antigen peaked during intermediate helminth infection but was comparable between acute and chronic helminth infection. Inhibition of airway inflammation could be transferred to OVA-sensitized recipient mice by B cells and $CD4^+$ T cells from spleens of chronically, but not acutely, infected mice. This protective effect was reversed when neutralizing anti-IL-10R antibodies were applied indicating a critical role for both B and T cell-derived IL-10.[155]

Subsequent studies have focused on the mechanisms involved in B cell-mediated suppression of OVA-induced allergic airway inflammation. Using a similar model, another group set out to identify and characterize the helminth-induced B cell population that mediates suppression of allergic airway inflammation. Spleen-derived IL-10-producing B cells from *Schistosoma mansoni*-infected mice expressed increased levels of CD1d compared to total B cells from infected mice. Furthermore, CD5, CD21 and IgM expression were increased in IL-10^{+}CD1d^{hi} B cells while CD23 and IgD expression were lower. B cells expressing low (non-Breg) or high (Breg) levels of CD1d were sorted from splenocytes of helminth-infected mice. Adoptive transfer of CD1d^{hi} but not CD1d^{lo} B cells to OVA-sensitized recipients could prevent and reverse allergic airway inflammation in an IL-10-dependent manner. Furthermore CD1d^{hi} B cells induced pulmonary infiltration of FoxP3+ Treg cells.[156]

In order to determine which organs are involved in *Schistosoma mansoni*-mediated protection against OVA-induced allergic airway inflammation, B cells isolated from lungs, mesenteric lymph nodes or spleens from OVA-sensitized helminth-infected mice were adoptively transferred to OVA-sensitized mice. Both lung-and spleen-derived B cells suppressed allergic airway inflammation. Interestingly, only suppression of inflammation by splenic B cells (of which CD1d^{+} cells were the main source of IL-10) was reversed upon anti-IL-10R mAb treatment indicating that lung-derived B cells utilize and IL-10-independent mechanism to suppress allergic airway inflammation. Similar to the findings by Amu *et al.*[156] an increase in pulmonary FoxP3^{+} Treg cells was observed after adoptive transfer of splenic B cells. *In vivo* depletion of FoxP3^{+} Treg cells only partially reversed splenic B cell-mediated protection against allergic airway inflammation indicating that Treg cells are not the main subset involved in protection against allergic airway inflammation. Interestingly, the frequency of IL-10^{+}CD1d^{hi} B cells in peripheral blood of *Schistosoma heamatobium*-infected children was increased compared to uninfected children.[157]

5.7.4 Regulatory B cells in humans

In analogy to the mouse models used to study regulatory B cells, human regulatory B cells have been mainly studied in relation to autoimmune diseases such as SLE and multiple sclerosis. It was found that CD40L stimulation of peripheral B cells (using human CD40L-transfected murine fibroblasts) induced IL-10 production primarily in $CD27^-$ naïve B cells. The frequency of IL-10-producing B cells and the amount of secreted IL-10 was strongly reduced in MS patients when compared to healthy controls. The phenotype of IL-10-producing B cells was not further delineated in this study.[158] A human B cell population characterized as $CD19^+CD24^{hi}CD38^{hi}$ was shown to produce IL-10 in response to CD40L stimulation (using human CD40L-transfected Chinese hamster ovary cells). This B cell population also expressed CD5 and high levels of CD1d as was described in the murine system. Furthermore these cells could suppress Th1 differentiation. Addition of a combination of blocking anti-IL-10, anti-CD80 and anti-CD86 mAbs could fully reverse the suppression of Th1 differentiation. This indicates that this suppression was partially dependent on IL-10, as well as CD80 and CD86. In SLE patients $CD19^+CD24^{hi}CD38^{hi}$ B cells failed to produce IL-10 and suppress Th1 differentiation in response to CD40L stimulation but still produced IL-10 after CpG stimulation.[159]

Helminths may also play a role in the induction of human regulatory B cells. It was found that MS patients that were infected with helminths showed increased IL-10 production by B cells in response to anti-CD40-coated murine fibroblasts. These $IL\text{-}10^+$ B cells were characterized as $CD5^-CD27^+IgD^+CD11b^-CD1d^{hi}$. In infected patients the amount B cell-derived IL-10 production increased to levels comparable to what was observed in healthy individuals. In order to identify co-stimulatory factors required for IL-10 production by B cells, co-cultures of myelin basic protein- and myelin oligodendrocyte glycoprotein-primed specific T cell lines with purified B cells were performed and the frequency of IL-10-producing B cells was measured by intracellular IL-10 staining. Addition of blocking anti-B7RP-1 mAb reduced the frequency of $IL\text{-}10^+$ B cells by approximately 75%. Blocking anti-CD40L or

anti-CD80/CD86 mAbs reduced the IL-10^{+} B cell frequency by approximately 30%. It must be noted here that the frequency of IL-10^{+} B cells that was measured in the T-B cell co-cultures was around 40%-50% of total B cells. This is much higher than what was observed in other human studies where frequencies of Breg cells were typically <5% of total B cells.[159-161].

Another study described human IL-10-competent B cells that were found primarily among CD24^{hi}CD27^{+} B cells. In this study IL-10 was measured by intracellular staining after two different regimens of stimulations. The first stimulation regimen consisted of 5 hours stimulation with LPS or CpG together with PMA/Ionomycin and Brefeldin A or Monensin. Cells that were stained positive for IL-10 after this stimulation were referred to as B10 cells and represented around 0.6% of the peripheral B cells. The second stimulation regimen consisted of 48 hours stimulation with LPS or CpG with or without recombinant CD40L followed by 5 hours stimulation with PMA/Ionomycin and Brefeldin A. Cells that were stained positive for IL-10 after this stimulation were referred to as B10 + progenitor-B10 (B10pro) cells and represented around 5% of the peripheral B cells. The highest frequency of IL-10^{+} B cells was observed after 48h of CpG + CD40L + 5h of PMA/Ionomycin and Brefeldin A stimulation. B10 and B10pro cells were predominantly found within the CD24^{hi}CD27^{+} subpopulation. CD24^{hi}CD27^{+} B cells were able to suppress monocyte cytokine production in an IL-10-dependent manner. The mean frequency of B10 + B10pro cells was significantly increased in patients with different autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren syndrome, autoimmune vesiculobullous skin disease, or multiple sclerosis compared with healthy controls.[161]

To investigate whether IL-10 and Bregs play a role in viral infection. A cohort of chronic hepatitis B patients undergoing spontaneous flares of liver disease was studied. The frequency of IL-10-producing B cells was measured by intracellular cytokine staining after 4d stimulation with CpG followed by 4 hours of stimulation with PMA/Ionomycin/Brefeldin A. Chronic hepatitis B

patients had approximately two-fold higher frequencies of IL-10-producing B cells than healthy controls. IL-10 production in response to CpG was not restricted to a single B cell subset but all B cell subsets were producing IL-10. IL-10 staining without any stimulation was used to identify the *in vivo* circulating IL-10-producing B cells. The majority of these cells were $CD24^{hi}CD38^{hi}$ immature B cells. However, the extremely low frequencies of IL-10 positive cells that could be detected this way (0.02%-0.04%) and the fact that data from only one patient are shown make it difficult to judge the value of these data. Furthermore, the frequency of $CD24^{hi}CD38^{hi}$ B cells increased during flares of liver disease while frequencies of other B cell subsets did not correlate with disease flares. Depletion of $CD24^{hi}CD38^{hi}$ B cells from PBMC by FACS resulted in a 2-4-fold increase in IFN-γ-producing $CD8^+$ T cells in response to stimulation with hepatitis B virus-derived peptides. When sorted $CD24^{hi}CD38^{hi}$ B cells were added back to hepatitis B virus peptide-stimulated PBMC, the IFN-γ-production by $CD8^+$ T cells was fully suppressed and could be restored by addition of anti-IL-10R blocking mAb. These data suggest that IL-10-producing B cells can suppress antiviral $CD8^+$ T cell responses and therefore implicate a pathogenic role for Bregs and IL-10 in chronic hepatitis B infection.[160]

5.7.5 B cell depletion therapy

Support for a relevant role in immune homeostasis mediated by regulatory B cells in humans comes from patients that underwent B cell depletion therapy using the anti-CD20 antibody Rituximab. This therapy is used to treat certain leukemias and lymphomas as well as autoimmune diseases including MS and RA.[162-164] Rituximab is currently also regarded as a promising drug to prevent antibody-mediated rejection of transplanted solid organs.[165] Besides its relatively high success rate (around 80% of treated rheumatoid arthritis patients show clinical improvement), there have been several reports that B cell depletion therapy leads to exacerbation of ulcerative colitis and development of psoriasis.[166, 167] This indicates that in certain cases the depletion of B cells can lead to pathogenesis and supports a functional role for regulatory B cells in humans.

5.7.6 Phenotype and isolation strategy for regulatory B cells

Interestingly, there are different subsets of B cells that are capable of producing IL-10. This is strongly dependent on the stimulation conditions to which the cells are exposed. Murine B cells express TLR4 and respond to LPS while human B cells lack TLR4 expression and are therefore unresponsive to LPS. Stimulation of murine B cells with TLR2-L and TLR4-L induced IL-10 and IL-6 production in MZ B cells while follicular B cells respond with IL-6 and IFN-γ production. Subsequent addition of agonistic anti-CD40 antibody boosted TLR-L-induced cytokine production.[168] The two major stimulants that induce IL-10 production by human B cells are CD40L and CpG. As mentioned earlier, there are several reports that describe helminth-derived antigens that may induce IL-10 production by B cells.

The general picture that arises from the different published studies on IL-10-producing B cells is that there are multiple subsets of B cells that can produce IL-10. The main murine B cell subsets that produces IL-10 in response to CD40 stimulation are MZ-like B cells ($CD19^{+}CD1d^{hi}CD21^{hi}CD23^{-}CD24^{hi}IgM^{hi}IgD^{lo}$) and T2-MZP B cells ($CD19^{+}CD1d^{hi}CD21^{hi}CD23^{hi}CD24^{hi}IgM^{hi}IgD^{hi}$).[169] Human B cells that produce IL-10 in response to CD40 stimulation have been characterized as immature transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}CD5^{+}CD1d^{hi}$).[159] It must be stressed that the above-mentioned combinations of surface markers are not exclusively expressed by IL-10-producing B cells. There is merely an enrichment of cells that produce IL-10 in response to CD40 stimulation within these subpopulations of B cells. In contrast, TLR-L-induced IL-10 production is not confined to immature B cells. This is true both in the murine and human system. A summary of reported surface markers that have been associated with regulatory B cells is listed in table 2.

CD1d expression has been used in both the human and mouse system to distinguish IL-10-producing B cells form non-IL-10-producing B cells. The

typical gating strategy is to sort CD1d^{hi} B cells (in some cases combined with gating on CD5^{+} B cells) as IL-10-producing B cells and CD1d^{lo} B cells as non-IL-10-producing B cells. There are some limitations to this approach. CD1d^{hi} B cells do not form a separate population and therefore gating of different cell populations remains somewhat arbitrary. Furthermore, even though IL-10-producing B cells are enriched among CD1d^{hi} B cells, only a fraction of the IL-10-producing B cells expresses CD1d^{hi} while the majority of IL-10-producing B cells do not express high levels of CD1d. This means that sorting CD1d^{hi} B cells allows enrichment of IL-10-producing cells but on the other hand bypasses a large fraction of IL-10-producing B cells because they do not express CD1d. The same applies to other surrogate markers that have been proposed to target regulatory B cells. Therefore the best strategy for the isolation of IL-10-producing B cells is sorting based on IL-10 secretion. This is technically challenging but provides the only means to obtain the pure population of all IL-10-producing cells.

Table 2. Reported markers for human and murine regulatory B cells

Marker	Function	Expression level on IL-10-producing B cells	Expressed on murine or human regulatory B cells
CD1d	Presentation of lipid antigens	High	Both
CD5	Ligand for CD72 involved in B-T cell interaction	Positive	Both
CD19	BCR co-receptor	Positive	Both
CD21	BCR co-receptor	Positive	Murine
CD23	Low-affinity IgE receptor	Both high and low expression levels reported on different IL-10$^+$ subsets	Both
CD24	Highly expressed on B cell progenitor cells and mature resting B cells. Functions as co-stimulatory molecule for T cell activation	High	Both
CD27	Binds to CD70. Functions as co-stimulatory molecule for T cell activation. Used as marker for memory B cells	Both high and low expression levels reported on different IL-10$^+$ subsets	Human
CD38	Expressed at both early and late stages of B and T-cell maturation. Marker for activation; monitors intracellular calcium levels. Also detected on erythroid and myeloid progenitors in bone marrow.	Both high and low expression levels reported on different IL-10$^+$ subsets	Human
IgD	BCR expressed on mature naïve B cells.	Both high and low expression levels reported on different IL-10$^+$ subsets	Murine
IgM	BCR expressed on mature naïve B cells.	High	Both
IL-10	Anti-inflammatory cytokine	High	Both

5.8 Scope of the thesis

This thesis provides novel insights into the role of B cells in the regulation of immune responses. The first part of the data presented in this thesis is focused on the functional and phenotypical characterization of inducible IL-10-producing B cells, which we designated Br1 cells (Chapter 6.1). The fact that certain B cells have the capacity to produce IL-10 combined with the notion that IL-10 can potently augment IgG4 production by B cells led us to investigate human IL-10-producing B cells and their link with immunoglobulin production. Furthermore we investigated the effect of SIT on allergen-specific IgG4 and IgE production and the frequency of allergen-specific Br1 cells.

Next we studied the effect of IL-10 overexpression on the function and phenotype of B cells (Chapter 6.2). We found that IL-10 overexpression induces a regulatory phenotype in B cells and arms them to suppress antigen-specific proliferation of PBMC, production of inflammatory cytokines and maturation and differentiation of monocyte-derived dendritic cells.

Tissue injury can lead to the release of host cell DNA, which is capped by telomeres containing large numbers of TTAGGG repeats. These hexamer nucleotide repeats have immunosuppressive capacity and may have therapeutic potential for treatment of chronic inflammatory diseases.[170] We studied the modulation of human B cell proliferation, differentiation and immunoglobulin production by mammalian telomeric DNA sequences containing immunosuppressive TTAGGG motifs (Chapter 6.3).

6 Results

6.1 IgG4 production is confined to human IL-10-producing B regulatory cells that suppress antigen-specific immune responses.

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Abstract

Background: IL-10-producing regulatory B cells suppress immune responses and lack of these cells leads to exacerbated symptoms in mouse models of chronic inflammation, transplantation and chronic infections. IgG4 is a blocking antibody isotype with anti-inflammatory potential that is induced in human high-dose antigen tolerance models.

Objective: To characterize human inducible IL-10-secreting B regulatory 1 (Br1) cells and to investigate their immunoregulatory capacity through suppression of cellular immune responses and production of anti-inflammatory immunoglobulins.

Methods: Highly purified IL-10-secreting B cells were phenotypically and functionally characterized by whole genome expression analysis, flow cytometry, suppression assay and antibody production. B cells specific for the major bee venom allergen phospholipase A_2 were isolated from beekeepers who displayed tolerance to bee venom antigens and allergic patients before and after specific immunotherapy.

Results: Human IL-10^+ Br1 cells expressed high surface CD25 and CD71, and low CD73. Sorting of $CD73^-CD25^+CD71^+$ B cells allowed enrichment of human Br1 cells that produced high levels of IL-10 and potently suppressed antigen-specific $CD4^+$ T cell proliferation. IgG4 was selectively confined to human Br1 cells. B cells specific for the major bee venom allergen phospholipase A_2 isolated from non-allergic beekeepers show increased expression of IL-10 and IgG4. Furthermore, the frequency of IL-10^+ PLA-specific B cells increased in allergic patients receiving allergen specific immunotherapy.

Conclusion: Our data show the characterization of IL-10^+ Br1 cells and *in vivo* evidence for two essential features of allergen tolerance; the suppressive B cell and IgG4-expressing B cell that are confined to IL-10+ Br1 cells in humans.

Key messages

- Human IL-10-secreting Br1 cells potently suppress antigen-specific T cell responses.
- Human Br1 cells can be isolated based on IL-10 secretion and surface expression of CD25 and CD71 and CD73.
- IgG4-producing antibody forming cells essentially develop from Br1 cells.
- IgG4 producing and IL-10 positive Br1 cells increase in allergen-SIT and high dose venom exposure in non-allergic beekeepers.

Capsule summary: IL-10-secreting Br1 cells suppress antigen-specific T cell proliferation and selectively upregulate IgG4 production demonstrating a dual anti-inflammatory role for Br1 cells. Allergen-specific Br1 cells are increased during allergen-SIT and high dose bee venom tolerance.

Key words: Immune tolerance, regulatory B cells, IL-10, IgG4

Abbreviations: BCR: B cell receptor, Br1: B regulatory 1, CFSE: Carboxy-fluorescein succinimidyl ester, CSR: class switch recombination, L: ligand, PBMC: peripheral blood mononuclear cell, PLA: phospholipase A_2, PPD: purified protein derivative, SIT: specific immunotherapy, TLR: toll like receptor, Tr1: T regulatory 1, Treg: regulatory T

Introduction

Peripheral tolerance to allergens utilizes multiple mechanisms to suppress allergic inflammation, including suppression of dendritic cells that support the generation of effector T cells; induction of dendritic cells that support the generation of regulatory T (Treg) cells; suppression of effector T cells; suppression of mast cells, basophils and eosinophils; interaction with resident tissue cells and remodeling; suppression of allergen-specific IgE and induction of allergen-specific IgG4.[171] Peripheral tolerance as a result of high-dose allergen exposure as occurs in beekeepers, cat owners or helminth-infected individuals is accompanied by increased amounts of specific IgG4 antibodies.[115, 172, 173] Because of its unique structural features in the hinge region, IgG4 has several non-inflammatory properties including low affinity for the classical Fcγ receptors and the capacity to form bi-specific, functionally monovalent, antibodies through Fab arm exchange.[75] IgG4 antibodies lack the ability to cross-link antigen and form immune complexes. In addition, IgG4 is unable to activate the complement cascade.[72, 174]

Studies on the mechanisms of immune responses to allergens have demonstrated that inducible Tr1 cells are dominant in healthy individuals.[101, 102] Tr1 cells not only suppress Th1 or Th2 cell responses but also have a direct influence on B cells. Tr1 cells as well as natural $CD4^+CD25^+$ Treg cells suppress IgE and induce IgG4. Both subsets of regulatory T cells (inducible and natural) reduce the IgE-secreting plasma cell frequency and simultaneously augment the IgG4-secreting plasma cell frequency.[65] A useful tool to study the *in vivo* regulation of immune tolerance in response to high-dose antigen exposure in humans is the beekeeper model.[102] Continuous exposure to high doses of bee venom antigens in non-allergic beekeepers diminishes T cell-mediated cutaneous late-phase swelling reactions, suppresses allergen-specific T cell proliferation and induces an *in vivo* switch from allergen-specific Th1 and Th2 cells toward IL-10-producing Tr1 cells.[102] Although they produce IgE antibodies against venom antigens, beekeepers do not show anaphylaxis. Furthermore beekeepers have high levels of circulating IgG4 antibodies specific for the major bee venom allergen phospholipase A_2 (PLA).[103, 175] Bee venom allergic patients on the other hand

develop IgE antibodies mainly to PLA, but show increased production of specific IgG4 in response to bee venom specific immunotherapy (SIT).[176]

B cells contribute to immune responses essentially through antigen presentation to T cells, secretion of cytokines and production of antibodies after differentiation to plasma cells.[134] When they receive the right survival signals plasma cells can reside for many years in dedicated niches in the bone marrow and continuously produce antibodies independent of antigen exposure.[49] Upon activation IgM^+IgD^+ naïve B cells may undergo class-switch recombination (CSR) leading to the expression of IgA, IgG or IgE antibodies. Human B cells express several toll like receptors (TLR) including TLR1, 6, 7, 8, 9 and 10. TLR7 (activated by single stranded RNA) and TLR9 (activated by hypomethylated CpG DNA) are the highest expressed TLRs on B cells.[23]

IL-10 is a key regulator of inflammatory responses and protects the host from tissue damage as a result of excessive inflammation.[62] It suppresses antigen presentation and the production of pro-inflammatory chemokines and cytokines. On the other hand, IL-10 enhances survival, proliferation, differentiation and isotype switching of human B cells.[62] IL-10 augments IgG4 production, whereas it inhibits IL-4-induced IgE CSR.[63, 64] IL-10-mediated immunosuppressive functions of B cells have been described in murine models of autoimmunity[137, 146, 177, 178], infection[154, 179], cancer[180] and allergic airway inflammation.[156, 181] The relevance of immunoregulatory functions of human B cells was illustrated in rheumatoid arthritis patients treated with the B cell depleting antibody rituximab who showed exacerbation of ulcerative colitis and development of psoriasis.[166, 167]

We hypothesize that if a B cell plays an anti-inflammatory role, the antibody isotype produced by the plasma cell originating from this B cell should also be anti-inflammatory. In the present study, we characterized human inducible IL-10-producing B cells which we designate B regulatory 1 (Br1) cells and their immunoglobulin production. Whole genome microarray expression analysis was performed to characterize human circulating Br1 cells. Functional experiments demonstrated that Br1 cells could directly suppress antigen-

specific $CD4^+$ T cell responses and significantly upregulate IgG4, but not IgG1, IgA or IgE upon differentiation to plasma cells. High-dose bee venom-exposed beekeepers and bee venom allergic patients before and after bee venom specific immunotherapy (SIT) were used to demonstrate these findings *in vivo*.

Materials and Methods

Study group. Heparinised peripheral blood samples form healthy donors and beekeepers as well as allergic patients before and after ultra rush bee venom SIT were employed in the study. For staining of IL-10 secreting PLA^- and PLA^+ B cells four allergic patients aged 18 to 65 years with a history of moderate-to-severe systemic allergic reactions to honeybee stings grade II to IV, positive intracutaneous skin tests to BV of less than or equal to 1024 g/ L, and BV-specific serum IgE levels of 0.7 kU/L or greater in the Immuno-CAP FEIA were included in the study. Details on the immunotherapy protocol have been described elsewhere.[182] Blood samples were taken before the start of therapy and on day 110. The study was approved by the ethical commission of the Canton of Graubünden, Switzerland and by the Ethical Commission of the Canton of Bern, Switzerland.

Isolation of PBMC, B cells and IL-10-secreting B cells. PBMCs were isolated by Ficoll (Biochrom, Berlin DE) density gradient centrifugation of peripheral venous blood. Cells were washed three times and resuspended in RPMI 1640 medium supplemented as previously described.[101] For experiments with purified B cells, untouched $CD19^+$ cells were isolated by labeling non-B cells using a cocktail of biotin-conjugated antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a and anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by immunomagnetic separation (AutoMacs; Miltenyi Biotec). The purity of isolated $CD19^+$ B cells was routinely >98% (Figure E1, A in the Online Repository). For some experiments pre-enriched B cells were labeled with anti-CD27-PE and $CD27^-$ and $CD27^+$ B cells were sorted by flow cytometry. For the isolation of alive IL-10^+ B cells, purified peripheral B cells were stimulated with 1 µM CpG 2006 (Microsynth GmbH, Balgach, Switzerland). After 3 d culture at 37° C in

humidified 5% CO_2, cells were harvested and labeled with 50 µg/ml anti-IL-10/CD45 antibody-antibody conjugates (Miltenyi Biotec) for 10 min at a concentration of 10^8 cell/ml in ice-cold supplemented RPMI 1640 medium. The cells were diluted with 37° C medium to a final concentration of 10^5 cells/ml and were allowed to secrete and capture IL-10 for 2 hours at 37° C. After capturing the secreted IL-10 on their surface, cells were centrifuged at 300x*g* for 5 min at 4°C and resuspended at a concentration of 10^8 cells/ml in ice-cold buffer containing 0.5% BSA and 2 mM EDTA (Sigma-Aldrich, St. Louis, MO USA) in PBS. Control cells were stained without the CD45/IL-10 antibody/antibody conjugate. The cells were then stained with 5 µg/ml PE-conjugated anti-IL-10 for 10 min at 4°C and washed and resuspended in BSA-EDTA PBS. Dead cells were excluded using 7-AAD and IL-10^+ and IL-10^- B cells were sorted using a FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ USA).

Cell cultures. Cells were cultured in RPMI 1640 medium supplemented as previously described.[101] Unless otherwise indicated cells were cultured at a density of $1x10^6$/ml. The following reagents were used for stimulation of B cells: 1 µM of the TLR7-L 3M-013 (3M Pharmaceuticals, St Paul MN, USA), 1 µM of synthetic phosphorothioate B type TLR9-L CpG 2006 (Microsynth), 10µg/ml of rabbit anti-human IgG + IgM (Jackson ImmunoResearch Europe, Suffolk, UK) for BCR stimulation, soluble CD40L ligand (sCD40L) was used as described previously[65], 100 U/mL of IL-2 (Proleukin ProreoPharma, Liestal, Switzerland), 50 ng/ml of IL-10 (PeproTech, London, UK), 25 ng/ml of IL-7, IL-9, IL-15 and IL-21 (PeproTech, London, UK).

Flow cytometry. The following antibodies and staining reagents were used: CD19-ECD, CD19-PC5, CD25-PC5, CD3-PC5, CD16-PC5, CD14-PC5 (Beckman Coulter, Fullerton, CA USA), IgM-PerCP/Cy5.5, CD5-PC7, CD24-AF488, CD38-PC7, CD73-APC, CD71-FITC, CD19-APC/Cy7 (Biolegend, San Diego, CA USA), CD27-PE, IgD-FITC, CD274-FITC (BD Biosciences), CD25-PE (DAKO, Glostrup, Denmark). PLA was labeled with FITC or Alexa Fluor-647. Matching isotype controls were used as negative controls. Samples were measured with a FACSAria II instrument (Beckton Dickinson) or Galios flow

cytometer (Beckman Coulter) and analyzed using Kaluza software (Beckman Coulter).

RNA isolation and cDNA synthesis. RNA was isolated using RNeasy kits (Qiagen, Hilden, DE). Reverse transcription was performed with reverse-transcription reagents (Fermentas, St. Leon-Rot, DE) with random hexamers according to the manufacturer's protocol.

Real-time PCR. cDNAs were amplified using SYBR green PCR master mix (Bio-Rad Laboratories, Hercules, CA USA) according to the manufacturer's recommendations using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA). Relative quantification was performed using the comparative ΔΔCT method.[183] The housekeeping gene EF1α was used for normalization. Figure 4b shows $2^{-(\Delta CT)}$ values multiplied such as to set the average expression level of IL-10^- cells to 1, where ΔCT corresponds to the difference between the CT value for the gene of interest and EF1α. Primers used: EF1a fw 5'-CTGAACCATCCAGGCCAAAT-3' rv 5'-GCCGTGTGGCAATCCAAT-3'; IL-10 fw 5'-GTGATGCCCCAAGCTGAGA-3' rv 5'-CACGGCCTTGCTCTTGTTTT-3'; IgE fw 5'-ACACATCCACAGGCACCAAA-3' rv 5'-TTGCAGCAGCGGGTCAA-3'; IgG4 fw 5'-ACC$^{C}/_{A}$TGGTCACCGTCTCCTCA-3' rv 5'-GGGACCATATTTGGACTC-3'; IgA fw 5'-CGCTGGCCTTCACACAGAA-3' rv 5'-CGCCATGACAACAGACACA-3'; IgG1 fw 5'-CTCTCAGCCAGGACCAAGGA-3' rv 5'-GGTGGGCATGTGTGAGTTTTG-3'; ; IL2RA fw 5'-AAACTCTAGCCACTCGTCCTG-3' rv 5'-ACTTGTTTCGTTGTGTTCCGA-3'; IL2RB fw 5'-GGACTTCAAGCCCTTTGAGAAC-3' rv 5'-TAGTGGGAGGCTTGGGAGAT-3'; JAK1 fw 5'-GGCATGCCGTATCTCTCCTC-3' rv 5'-GTGATGGTGCGATTTGGAGC-3'; STAT1 fw 5'-GTTATGGGACCGCACCTTCA-3' rv 5'-CATGCAGGGCTGTCTTTCCA-3'; STAT3 fw 5'-ACCAGCAGTATAGCCGCTTC-3' rv 5'-GCCACAATCCGGGCAATCT-3'; STAT5A fw 5'-GCAGAGTCCGTGACAGAGG-3' rv 5'-CCACAGGTAGGGACAGAGTCT-3'; TFRC/CD71 fw 5'-GGCTACTTGGGCTATTGTAAAGG-3' rv 5'-CAGTTTCTCCGACAACTTTCTCT-3'; PDL1/CD274 fw 5'-ACTGGGACATT

CGGGTTTTGA-3' rv 5'- CCTCACTTTCTGAGCGATGAGT-3'; CD73 fw 5'-ACTGGGACATTCGGGTTTTGA-3' rv 5'-CCTCACTTTCTGAGCGATGAGT-3'; PTPN11 fw 5'-AGAGAAAGGTGTTGACTGCGAT-3' rv 5'-TCCTGCGCTGT AGTGTTTCA-3'. Immunoglobulin mRNA expression was always measured after 5 d. Expression of differentially regulated genes in IL-10^- compared to IL-10^+ B cells that were identified by gene array were measured after 3 d (immediately after sorting IL-$10^-$and IL-10^+ cells).

Suppression assay. IL-10^+ and IL-10^- B cells were co-cultured with 2 x 10^5 autologous PBMC in ratios of B cells:PBMC of 1:100, 1:50 and 1:25. $CD73^-$ $CD25^+CD71^+$, $CD73^-CD25^-CD71^-$, $CD73^+CD25^+CD71^+$ and $CD73^+CD25^-$ $CD71^-$ B cells were sorted from resting B cells and subsequently stimulated for 48 hours with TLR9-L. Cells were then washed and co-cultured with 2 x 10^5 autologous PBMC in ratios of B cells:PBMC of 1:25 and 1:12. For CFSE dilution experiments PBMC were labeled with 5 µM CFSE (Invitrogen) and washed twice with supplemented RPMI 1640. As a control for proliferation without suppression no B cells were added in a control group. Cells were stimulated with 1 µg/ml PPD (Statens Serum Institut, Copenhagen, DK) and cultured in a 96-well plate. For [^{3}H]thymidine incorporation measurements cells were pulsed for 8 hours at 37° C with 1 µCi/well [^{3}H]thymidine (DuPont; New England Nuclear), and the incorporation of labeled nucleotide was measured in an LKB β plate reader (GE Healthcare). To determine CFSE dilution, cells were stained with anti-human CD4-APC (Beckton Dickinson) and analyzed using a Gallios flow cytometer (Beckman Coulter). IL-10R blocking antibodies and isotype control (Biolegend) were used at 5 µg/ml. rIL-10 (Peprotech) was used at a concentration of 25 ng/ml.

Quantification of cytokines and immunoglobulins. IL-10 was measured using ELISA as described previously[63] or the Bio-Plex Hu Cytokine Panel, 17-Plex Group 1 (Bio-Rad Laboratories). Human IgG1, IgG4, IgA, and IgE in cell culture supernatants were measured after a total 10d culture period using a Bio-Plex Pro™ Human Isotyping Panel (Bio-Rad Laboratories). Fluorescent signals were read and analyzed using the Bio-Plex 200 System (Bio-Rad

Laboratories). PLA-specific IgE and IgG4 from serum samples were measured by ELISA as previously described.[101]

Statistical Analysis. Unless otherwise indicated data show mean ± SEM. Statistical tests were performed using GraphPad Prism 5.0c. Paired *t*-tests and Wilcoxon signed rank tests were used for assessing statistical significance. The ratio of serum measurement of anti-PLA IgG4:IgE was analyzed by one-way ANOVA with Turkey posthoc test. Statistical analysis applied to gene array data is described in the methods section of the whole human genome microarray analysis. The grade of statistical significance is displayed in the legends to the figures. P values of <0.05 were considered significant.

Whole human genome microarray analysis. IL-10^- and IL-10^+ B cells from 6 healthy individuals were purified after 3 d TLR9-L stimulation. 5000 IL-10^+ and 5000 IL-10^- B cells were sorted using a FACSAria II cell-sorter (BD biosciences) and cells were lysed in SuperAmp Lysis Buffer (Miltenyi Biotec) following the manufacturer's instructions and stored at -80°C. A dual-color hybridization of human RNAs on Agilent Whole Human Genome Oligo Microarrays and bioinformatics analysis was performed by Miltenyi Biotec. Gene expression analysis using the Agilent platform (Agilent Technologies, Palo Alto, USA) was performed at Miltenyi Biotec's gene array facility (Bergisch Gladbach, Germany). RNA was isolated using paramagnetic oligo(dT) MicroBeads. Uniform-sized cDNA fragments were generated by an in-column cDNA synthesis procedure. All cDNA fragments were tailed at the 3'-end and amplified by single-primer global PCR. cDNA was quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies). The integrity of the amplified cDNA was determined using the Agilent 2100 Bioanalyzer platform (Agilent technologies). The average length of the highly amplified cDNA products ranged between 200–1,000 bp. 250 ng of each of the cDNAs were used as template for Cy3 and Cy5 labeling in a Klenow Fragment reaction. IL-10^+ samples were Cy5-labeled and IL-10^- samples were Cy3-labeled. The Cy3- and Cy5- labeled cDNAs were combined and hybridized overnight (17 hours, 65°C) to an Agilent Whole Human Genome Oligo

Microarrays 4 x 44K. Finally, the microarrays were washed once with 6x SSPE buffer containing 0.005% N-lauroylsarcosine for 1 min at room temperature followed by a second wash with pre-heated 0.06x SSPE buffer (37 °C) containing 0.005% N-lauroylsarcosine for 1 min. Fluorescence signals of the hybridized Agilent Oligo Microarrays were detected using a DNA microarray scanner (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. Preprocessing of the microarray data include normalization using the Lowess algorithm[184] and incorporation of the Rosetta error model.[185] The resulting ratios for each reporter between the signal intensities of the co-hybridized treated and untreated samples were transformed to logarithms to base 2 (log2 ratios). Individual signal detection- and ratio- p-values were calculated (Rosetta error model, Rosetta Inpharmatics LLC, Seattle, USA) to evaluate the reliability of signal, and expression based on signal to background intensities of both co-hybridized samples per array. Expression differences for individual reporters between the treated and untreated sample groups were identified by one-group t-test followed by multiple testing correction (Benjamini & Hochberg)[186] on the log2 ratio data and effect size (fold change). Candidate genes with differential expression were selected if they passed a corrected p-value ≤ 0.05 in the group-wise comparison. Genes were additionally filtered for detection p-value ≤ 0.01 in at least four out of six samples per channel, followed by selection of array data sets with ratio p-values ≤ 0.01 in all six replicate experiments. Candidate genes that passed these requirements are listed in Table E1 in the online repository.

Reporters identified in the discriminatory genes analysis were annotated with information from Gene Ontology (GO), which provides information on molecular function, as well as various pathway resources for information on involvement in biological signaling pathways (The Gene Ontology Consortium, 2000). All annotations were summarized and curated by the Miltenyi Bioinformatics team (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Signaling pathways and targets of pathways were additionally curated based on information from NetPath database[187]. For an assessment of the enrichment of a category, Fisher's exact test with Benjamini-Hochberg

correction for multiple testing[186] was performed. Values of ≤ 0.05 indicate a significant enrichment relative to the background (whole reporter-sets of the Agilent 4x44K Whole Human Genome Oligo Microarray) of the respective category.

Several genes encoding surface markers were differentially expressed (See Table E2 in the online repository). Differential expression of surface marker genes was based on alignment with a list of described genes encoding surface markers obtained from Immunome Knowledge Database.[188] Genes that were differentially expressed between IL-10$^-$ and IL-10$^+$ B cells were compared to a published list of genes that are regulated by IL-10 in human monocytes[189] (See Table E3 in the online repository). Genes that were differentially expressed between IL-10$^-$ and IL-10$^+$ B cells were compared to genes that were previously described to be regulated by TLR9-L in human B cells[190] (See Table E4 in the online repository). Microarray data are available under GEO accession no. GSE35002.

Results

Purification and characterization of human IL-10-secreting Br1 cells

Stimulation with the TLR9-L CpG2006 was found to optimally induce IL-10 in purified B cells. In order to purify alive IL-10-secreting (IL-10^+) B cells, stimulated B cells were labeled with anti-IL-10/CD45 antibody-antibody conjugates and subsequently incubated to allow secretion and capture of IL-10 to the cell surface. Both resting as well as proliferating B cells expressed IL-10 in response to TLR9 stimulation (See Figure E1, A-C in the Online Repository).

In order to characterize Br1 cells, IL-10^+ B cells and IL-10^- B cells were sorted and IL-10 mRNA expression was strongly confined to IL-10^+ cells (Figure 1A). Purified IL-10^+ and IL-10^- B cells were subjected to whole genome microarray expression analysis. Several genes involved in IL-2 signaling were upregulated in IL-10^+ cells including *IL2RA, IL2RB, JAK1, STAT1, STAT3, STAT5A, PTPN11* (See supplementary Figure E1, D and Table E1 in the Online Repository). JAK1 and STAT3 are also key factors in IL-10 signaling. Several genes encoding surface markers were differentially expressed between IL-10^- and IL-10^+ B cells. A heat map of the five of genes showing the highest median differential expression between IL-10^- vs. IL-10^+ genes is shown in Figure 1B. Genes encoding surface markers that showed higher expression in IL-10^+ cells included *CD80* (4.5-fold), *IL2RA* (4.4-fold), *TFRC* (4.1-fold), *CD274* (4.1-fold) and *IL2RB* (2.8-fold). Genes encoding surface markers that showed lower expression in IL-10^+ cells included *PTPRJ* (2.5-fold), *NT5E* (2.3-fold), *CD79B* (2.2-fold), *CD37* (2.0-fold) and *FCGR2B* (1.9-fold) (Figure 1B and Table E2 in the Online Repository). Differential expression of IL2RA (CD25), TFRC (CD71), CD274 (PD-L1) and NT5E (CD73) was confirmed by real-time PCR (Figure 1C) and flow cytometry (Figure 1D). Thus, IL-10^+ B cells showed elevated expression of CD25, CD71 and PD-L1 while CD73 expression was reduced. We then investigated the expression of other B cell markers and surface markers that have been linked to Breg cells in previous reports. Surface expression of CD38, CD24, CD5, CD27, IgM and IgD was also measured to further characterize IL-10^+ B cells. TLR9-L-induced IL-10^+ cells had similar frequencies among $CD24^{int}CD38^{int}$,

CD24^{hi} CD38^{-} and CD24^{hi} CD38^{hi} B cells (See Figure E1, E in the Online Repository). The frequency of CD27^{+} B cells was similar between IL-10^{-} and IL-10^{+} B cells while the level of CD27 expression was lower among CD27^{+} B cells in the IL-10^{+} population.

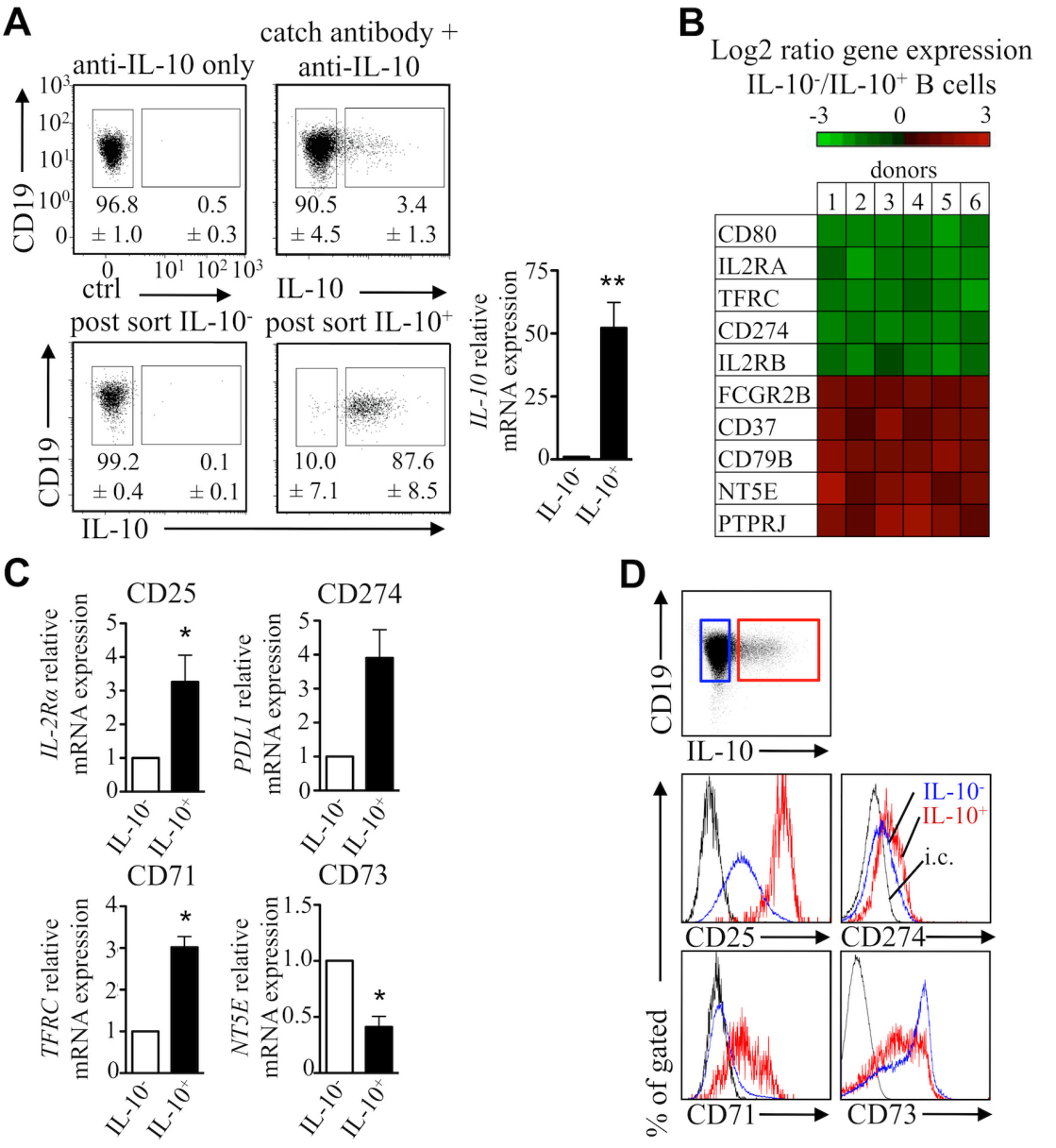


*Figure 1. Characterization of IL-10^{-} and IL-10^{+} B cells. (A) Sorting of IL-10^{-} and IL-10^{+} B cells. IL-10 expression was confirmed by real-time PCR. (B) Sorted fractions (n=6) (B) Gene array result showing up- or downregulated surface marker genes. (C) mRNA expression (relative to IL-10^{-}) of selected surface markers (n=6) (D) CD25, CD71, CD274 and CD73 surface staining (representative example out of 3). * P<0.05, ** P<0.01.*

The frequencies of $CD5^+$ as well as IgM^+IgD^- cells were two-fold higher in IL-10^+ cells (See Figure E1, F in the Online Repository). Again, purified $CD27^-$ and $CD27^+$ B cells produced similar levels of IL-10 in response to TLR9-L (See Figure E1, G in the Online Repository). These data suggest that B cells that produce IL-10 in response to TLR9-L stimulation are not restricted to a single subset of B cells.

IL-10-producing B cells are enriched in $CD73^-CD25^+CD71^+$ B cells

In order to isolate IL-10-producing B cells, we stained CD25 and CD71 as high expression markers and CD73 as a low/negative expression marker on circulating B cells without any stimulation. PD-L1 surface expression showed only a mild upwards shift in IL-10^+ B cells and therefore was not suitable as a distinguishing marker. Among $CD73^-$ B cells, $CD25^+$ and $CD71^+$ cells formed a separate population (See Figure E2 in the Online Repository) and $CD25^+CD71^+$ B cells had a 4-fold higher frequency in $CD73^-$ cells than in $CD73^+$ cells (Figure 2A). $CD73^-CD25^+CD71^+$, $CD73^-CD25^-CD71^-$, $CD73^+CD25^+CD71^+$ and $CD73^+CD25^-CD71^-$ B cells were sorted (Figure 2B) and subsequently stimulated with TLR9-L. We found that $CD73^-CD25^+CD71^+$ B cells secreted the highest amounts of IL-10. In contrast, $CD73^+CD25^+CD71^+$ cells secreted 3-fold less IL-10, and $CD73^-CD25^-CD71^-$ and $CD73^+CD25^-CD71^-$ cells showed the lowest IL-10 production (Figure 2C).

IL-10 plays an essential role in T cell suppression by human Br1 cells

Next, we wanted to determine whether Br1 cells have suppressive capacity on antigen-specific proliferative responses. IL-10^+ and IL-10^- B cells were purified using the IL-10-secretion assay and co-cultured at different ratios with autologous PBMC that were stimulated with PPD. With a ratio of 1 B cell to 25 PBMC, we observed up to 50% suppression by IL-10^+ B cells on PPD-induced proliferative responses in PBMC whereas other B cells did not show suppressive capacity. This effect was reversed upon blocking the IL-10 receptor. CFSE dye analysis confirmed these findings and furthermore demonstrated that proliferation of $CD4^+$ T cells in response to PPD stimulation was strongly suppressed by IL-10^+ B cells. PPD-induced $CD4^+$ T cell proliferation was suppressed around 50% with a ratio of one IL-10^+ B cell to

25 responder cells (Figure 3A and Figure E3 in the Online Repository). IL-10$^-$ B cells could also partially suppress CD4$^+$ T cell proliferation. In the presence of a blocking anti-IL-10R antibody the percentage of proliferating CD4$^+$ T cells was generally higher in cultures with added B cells (both IL-10$^-$ and IL-10$^+$) than in PBMC (Figure 3A) possibly due to increased antigen presentation by B cells and neutralization of endogenous IL-10.

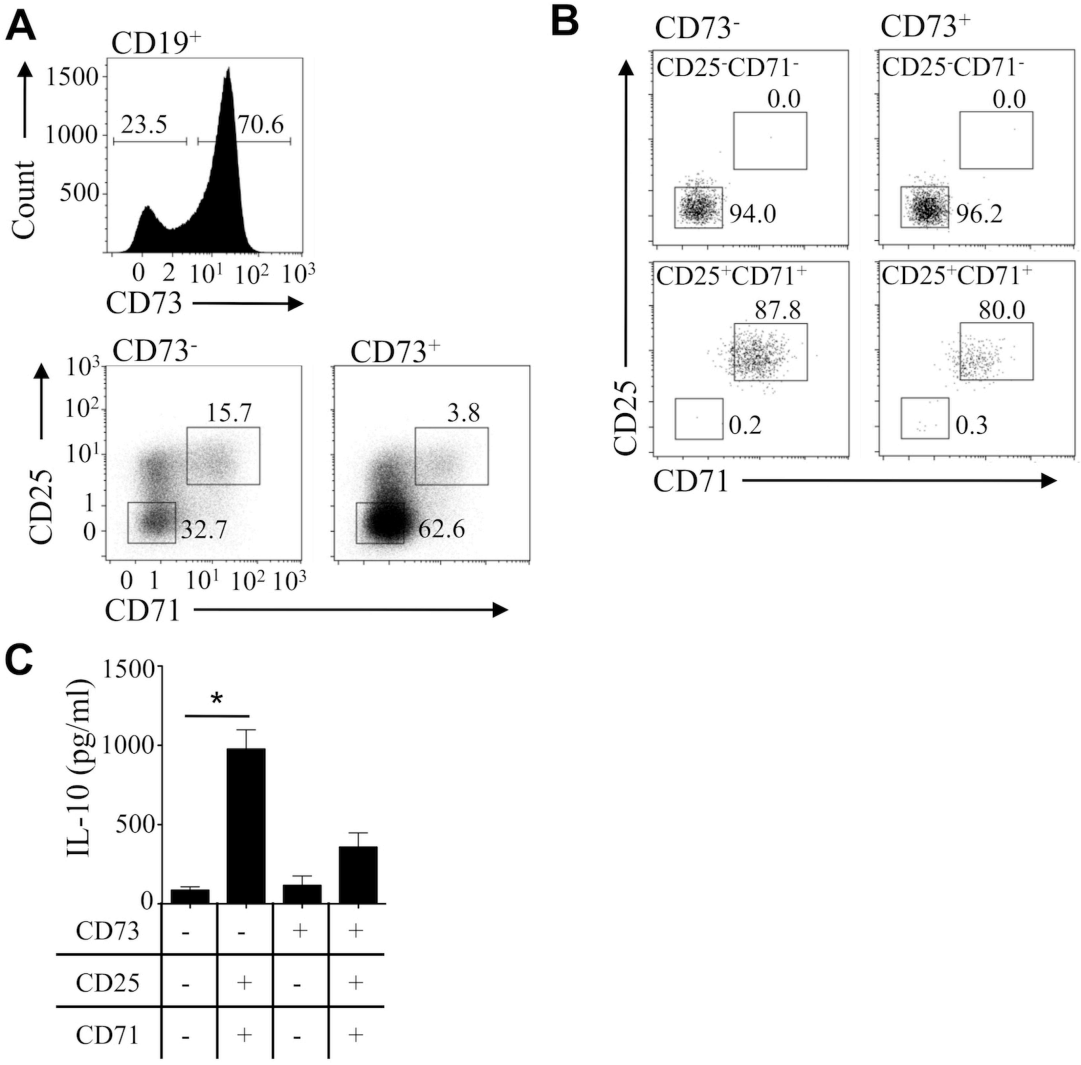


*Figure 2. Sorting of CD73, CD25 and CD71+/- populations from resting B cells. (A) Staining of CD25 and CD71 on CD73$^-$ and CD73$^+$ B cells. (n=3, one representative example is shown) (B) Sorted B cell populations were reanalyzed to assess purity. (C) Sorted B cell populations (n=3, 5x10^4 cells in 100µl) were stimulated for 48h and IL-10 was measured in supernatants. * P<0.05.*

To identify the suppressive capacity of B cells that differentially express CD25, CD71 and CD73, $CD73^{-}CD25^{+}CD71^{+}$, $CD73^{-}CD25^{-}CD71^{-}$, $CD73^{+}CD25^{+}CD71^{+}$ and $CD73^{+}CD25^{-}CD71^{-}$ B cells were sorted and co-cultured at different ratios with autologous PPD-stimulated PBMC. Only $CD73^{-}CD25^{+}CD71^{+}$ B cells could suppress $CD4^{+}$ T cell proliferation, whereas the other three B cell subsets did not show any suppressive effect (Figure 3B). This suppressive effect was however not fully reversed through addition of blocking anti-IL-10R antibody. Due to cell number limitations higher ratios of B cells to PBMC could not be used.

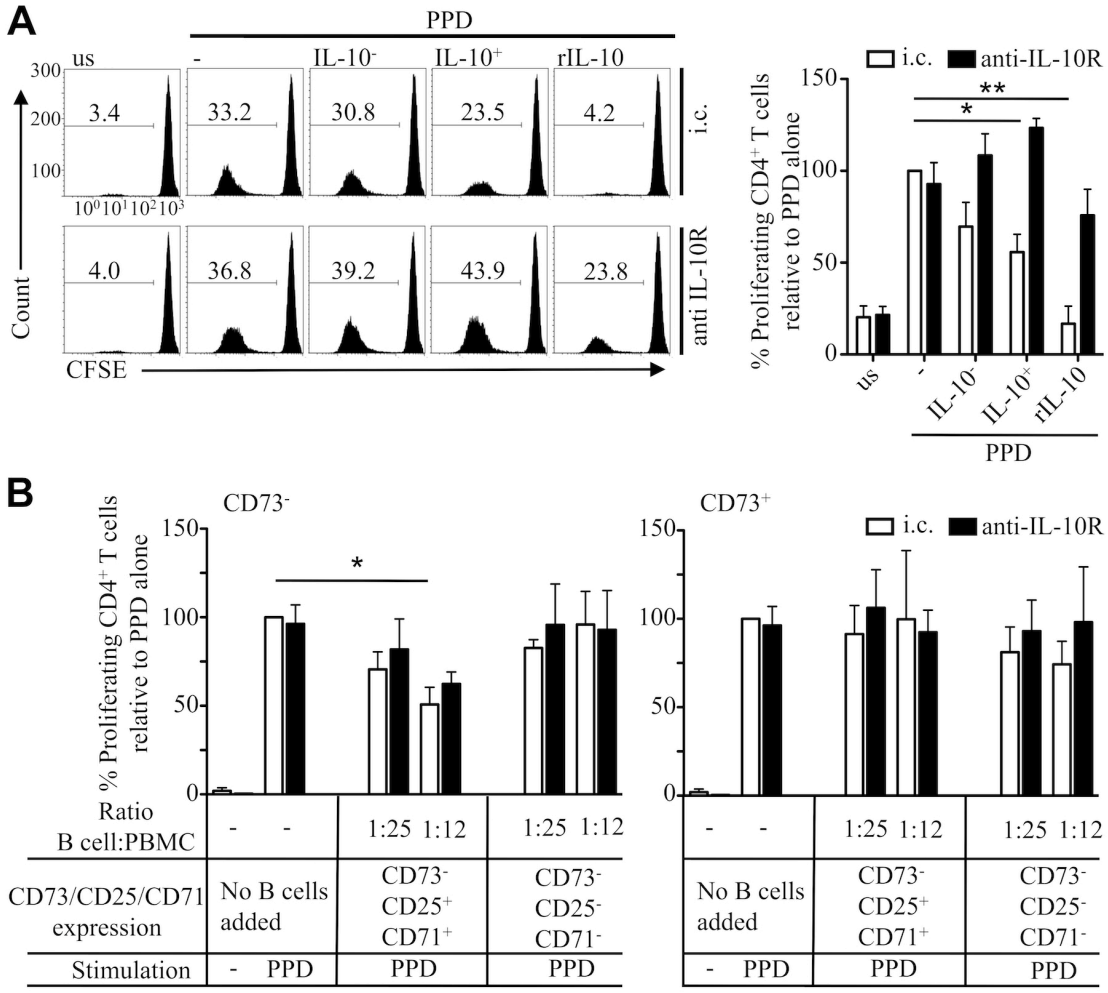


*Figure 3. Br1 cells suppress antigen-specific proliferation. (A) Co-cultures of CFSE-labeled PBMC with IL-10^{+} or IL-10^{-} B cells (ratio: 1 B cells to 25 PBMC) with blocking anti-IL-10R or i.c. mAbs. Recombinant IL-10 (rIL-10) was used as control. Histograms show representative example. Numbers indicate percentage dividing CD4^{+} cells. (B) Co-cultures of PBMC with CD73, CD25 and CD71+/- B cells. Combined data from four independent experiments is shown. * P<0.05, ** P<0.01.*

Immunoglobulin production of Br1 cells

We then investigated the isotype of the produced immunoglobulin by circulating human Br1 cells upon their differentiation to plasma cells. TLR9-L stimulation of purified B cells induced expression of IgG1, IgG4 and IgA, but not IgE at the mRNA level, whereas protein secretion of all four subclasses was induced. TLR9-L + IL-10 stimulation showed a synergistic effect on IgG4 production while IgG1 and IgE induction were not affected. IgA production showed a tendency to be suppressed by IL-10. (Figure 4A). The small amount of IgG4 induced by TLR9-L was mainly derived from $CD27^+$ B cells and was dependent on endogenous IL-10 production as blocking of IL-10R largely suppressed IgG4 production by $CD27^+$ cells. Dual stimulation with TLR9-L and IL-10 particularly increased IgG4 production in $CD27^-$ B cells (See Figure E4 in the Online Repository).

In the next step, $IL\text{-}10^-$ and $IL\text{-}10^+$ B cells were sorted to investigate their immunoglobulin profile upon activation with TLR9-L. $IL\text{-}10^-$ and $IL\text{-}10^+$ B cells were cultured for 5 d and the expression of immunoglobulin mRNAs were analyzed. IgG4 transcripts were expressed on average 10-fold higher in the $IL\text{-}10^+$ population compared to the $IL\text{-}10^-$ population (Figure 4B). IgG1, IgA and IgE were not differentially expressed between the two populations.

To further delineate this distinct upregulation of IgG4, $IL\text{-}10^+$ and $IL\text{-}10^-$ cells were isolated from $CD27^-$ and $CD27^+$ B cells that were sorted before stimulation with TLR9-L and immunoglobulin production was measured. $IL\text{-}10^-$ and $IL\text{-}10^+$ B cells derived from $CD27^+$ B cells showed no significant differences in the expression of IgG1, IgG4 or IgA. $IL\text{-}10^+$ B cells derived form $CD27^-$ B cells on the other hand produced 15-fold higher IgG4 than $IL\text{-}10^-$ cells whereas IgG1 and IgA production was not significantly increased (Figure 4C).

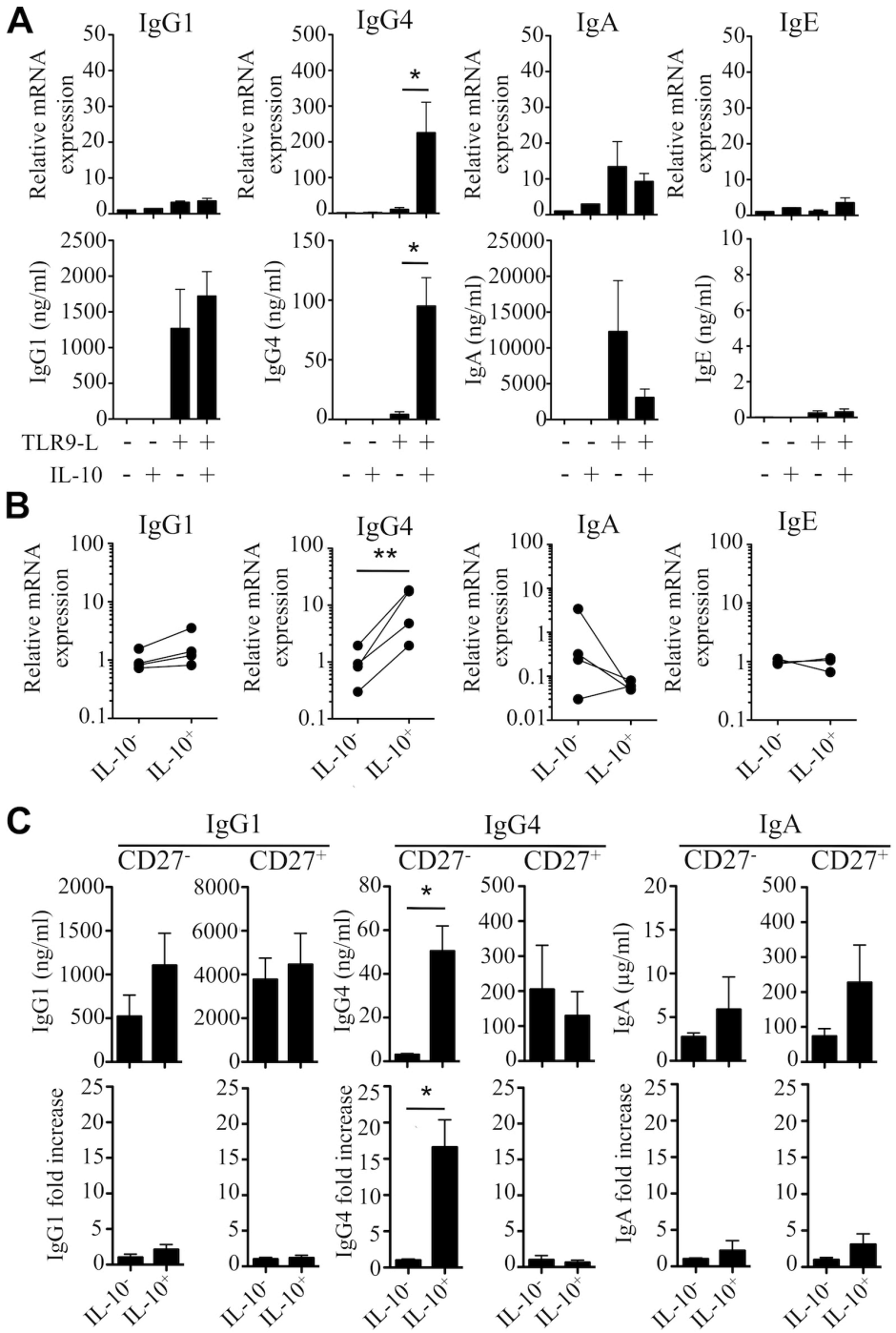


*Figure 4. Immunoglobulin production by Br1 cells. (A) IgG1, IgG4, IgA and IgE mRNA expression (relative to unstimulated cells) in total B cells. Lower panel: IgG1, IgG4, IgA measured in supernatants. (n=5, 3 independent experiments). (B) IgG1, IgG4, IgA and IgE mRNA expression (average IL-10$^-$ expression=1) in IL-10$^-$ and IL-10$^+$ B cells. Connected dots represent individual donors. (C) Secreted IgG1, IgG4 and IgA measured from IL-10$^-$ and IL-10$^+$ cells derived from CD27$^-$ and CD27$^+$ B cells (n=4). Fold increase is relative to average of IL-10$^-$. IgE protein was not detectable. *P<0.05, **P<0.01.*

Allergen-specific Br1 cells are confined to IgG4 production in high-dose bee venom tolerance

PLA-specific B cells from highly bee venom-exposed tolerant beekeepers were enriched by using labeled PLA. Between 0.1% and 0.9% of beekeeper-derived $CD19^+$ peripheral B cells were specific for PLA (Figure 5A). Subsequently, PLA-specific (PLA^+) and non PLA-specific (PLA^-) B cells were sorted (Figure 5A). Immunoglobulin isotypes and IL-10 mRNAs were measured immediately after sorting of the cells without any stimulation. IgG4 expression was significantly higher (median increase 14.1-fold) in PLA^+ B cells than in PLA^- B cells. IgA expression showed a modest decrease (median decrease 2-fold) in PLA^+ B cells whereas expression of IgE and IgG1 transcripts showed no significant difference between PLA^+ and PLA^- B cells. IL-10 mRNA expression was significantly increased (median increase 30.8-fold) in PLA-specific B cells (Figure 5B). To measure IL-10 protein production, B cells from beekeepers and bee venom allergic patients before and after SIT were stimulated for 3 d with CpG and stained for IL-10 and PLA-binding to their surface. The mean frequency of $IL\text{-}10^+$ cells was 1.8-fold higher in PLA^+-gated than in PLA^--gated B cells from beekeepers. PLA^+ B cells in allergic patients had relatively low frequencies of $IL\text{-}10^+$ B cells (1.8-2.9 %), which significantly increased (5.5-13.9 %) after 110 days of bee venom SIT. (Figure 5 C). In line with the flow cytometry data, secreted IL-10 in these samples was 2-fold higher in PLA^+ than in PLA^- cells. (Figure E5).

Additional *in vivo* evidence for the role of Br1 cells in allergen tolerance that links IL-10 to IgG4 was obtained by measuring of PLA-specific IgE and IgG4 in serum of beekeepers, bee venom allergic individuals and allergic individuals who received 110 days of bee venom-SIT. Allergic individuals who received SIT showed a significantly lower (100-fold) ratio of PLA-specific IgE:IgG4 when compared to non-treated allergic individuals. This difference was even greater (1000-fold) when compared to non-allergic beekeepers. In both cases this was due to substantially increased levels of specific IgG4. (Figure 5 D).

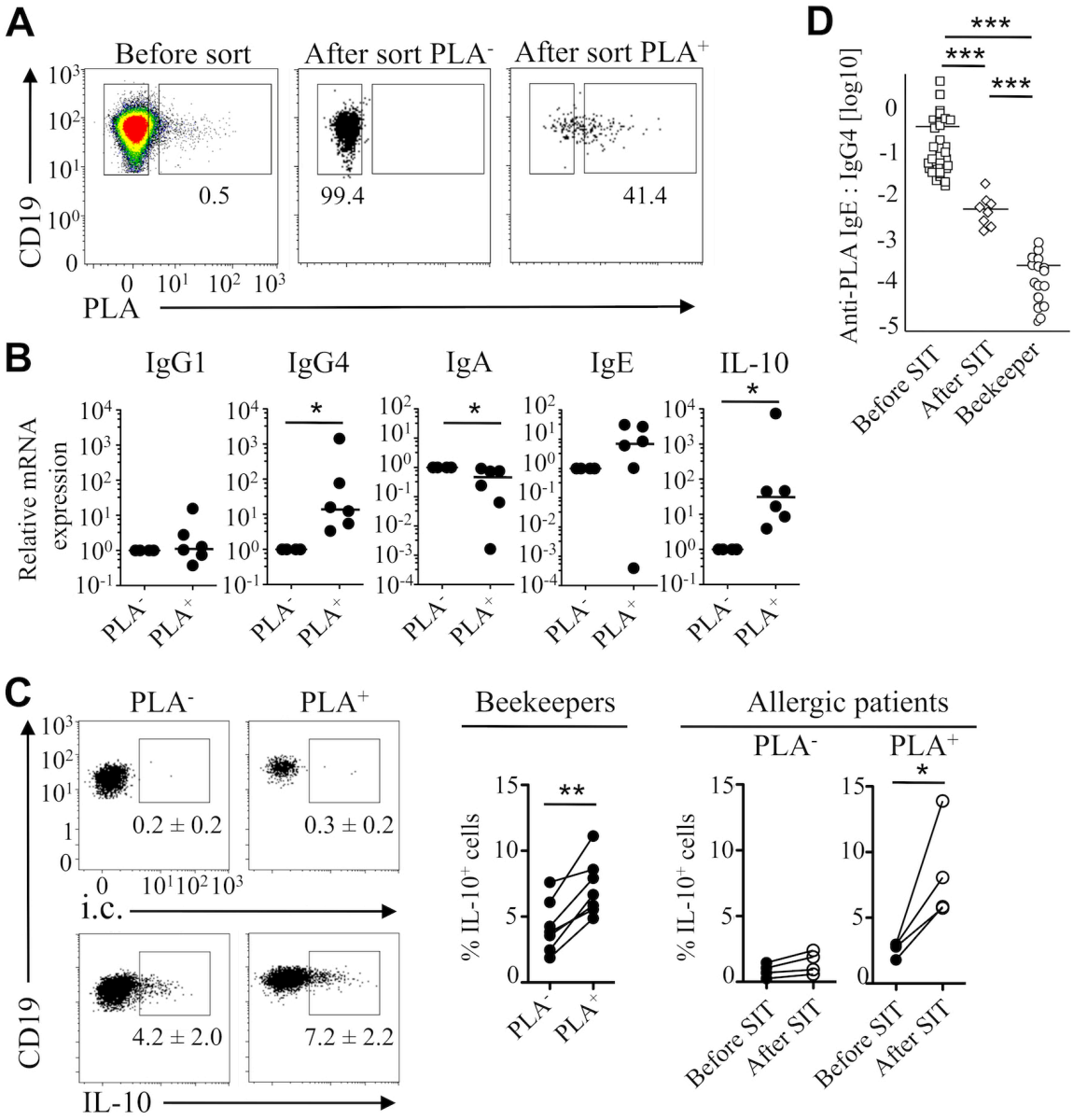


*Figure 5. Allergen-specific B cells from tolerant individuals upregulate IL-10 and IgG4. (A) Enrichment of PLA-specific B cells from beekeeper-derived peripheral CD19$^+$ cells. (B) Direct ex-vivo analysis of immunoglobulin and IL-10 mRNA expression (relative to PLA$^-$ cells). (n=6) Horizontal bars indicate median. (C) IL-10 and PLA staining in B cells from beekeepers (n=7) or bee venom allergic patients (n=4) after 3d TLR9-L stimulation. Numbers in dot plots: mean (± SD). (D) Ratio of PLA-specific serum IgE:IgG4. *P<0.05 **P<0.01, ***P<0.001.*

Discussion

This is the first study in which human inducible IL-10-producing B cells (designated as Br1 cells) were purified based on their secretion of IL-10. Gene arrays were performed and novel molecules that characterize Br1 cells were identified. We demonstrated a potent suppressive capacity of IL-10-producing B cells on antigen-specific $CD4^+$ T cell activation and, more importantly, we observed a striking shift towards production of anti-inflammatory IgG4 antibodies in these cells.

Despite accumulating data describing immunoregulatory functions of IL-10-producing B cell subsets in murine models, there is still little known about the function and phenotype of human regulatory B cells. There are indications that distinct B cell subsets have immunoregulatory functions in humans.[159, 166, 167] Human $CD24^{hi}CD38^{hi}$ B cells were shown to suppress the differentiation of Th1 cells through provision of IL-10 and possibly also through CD80- and CD86-mediated signaling.[159, 161] In another study, a $CD24^{hi}CD27^+$ human B cell subset was identified that contained the majority of IL-10-producing B cells and was found at higher frequencies in patients with autoimmune disease compared to healthy controls.[161]

A fraction of approximately 1-4 % of B cells secreted IL-10 in response to TLR9-L stimulation. These cells were not restricted to a single population of B cells since both $CD27^-$ (primarily naïve) and $CD27^+$ (primarily memory) B cells secreted similar amounts of IL-10. Unlike the described $CD24^{hi}$ $CD38^{hi}$ Breg cells that produced IL-10 in a CD40L-dependent manner[159],the frequency of Br1 cells was equally distributed among $CD24^{int}$ $CD38^{int}$, $CD24^{hi}$ $CD38^-$ and $CD24^{hi}$ $CD38^{hi}$ B cells. Furthermore $CD5^+$ and IgM^+IgD^- cells were enriched among $IL\text{-}10^+$ B cells. Using whole genome expression analysis and flow cytometry we could identify four surface markers that were significantly differently expressed on Br1 cells compared to $IL\text{-}10^-$ B cells. Br1 cells showed elevated expression of CD25, CD71 and CD274 whereas CD73 expression was lower. Some of these molecules are known to play a role in immune regulation. CD25 is expressed at high levels on natural Treg cells and contributes to their suppressive effect through cytokine deprivation-

mediated apoptosis of $CD4^+$ effector cells.[191, 192] Interestingly, CD25 expression appears to be essential for IL-10 expression by $CD4^+$ T cells and thereby plays an important role in the generation of Tr1 cells.[193] CD71 is the major transferrin receptor, which is involved in the cellular uptake of iron and in the regulation of cell growth. CD25 and CD71 are both known activation markers of T and B cells and their elevated expression may reflect an increased activation state.[194] CD274, also named PD-L1, is an inhibitory co-stimulatory molecule that counteracts T cells activation through binding to PD-1 on T cells.[195] PD-L1 can inhibit T cell responses by promoting both the induction and maintenance of inducible Treg cells.[196] CD73, alternatively named ecto-5'-nucleotidase, is a cell surface enzyme expressed on many cell types including T and B cells. CD73 converts adenosine monophosphate to adenosine, which has potent immunosuppressive effects. CD73 expressed on Treg cells may be responsible for part of their immunosuppressive effects.[197] Br1 cells on the other hand express low levels of surface CD73 and therefore adenosine-mediated immune-suppression is unlikely to play an important role in suppression of T cell responses.

$IL\text{-}10^+$ cells potently suppressed antigen-specific proliferation of $CD4^+$ T cells. A ratio of 1 B cell to 25 PBMC led to 50% suppression of the proliferative T cell response. $IL\text{-}10^-$ B cells could also weakly suppress proliferation. A possible explanation can be that the sensitivity of the IL-10 secretion assay was not sufficient to detect cells that produce minute amounts of IL-10 that may remain in the IL-10- fraction and render them slightly suppressive. Kinetics of IL-10 induction could also play a role as some cells may not secrete IL-10 at the time of sorting, but may do so later. The suppressive effect observed in our experiments is completely reversible through blocking of the IL-10 receptor. $CD73^-CD25^+CD71^+$cells sorted from resting B cells could similarly suppress T cell proliferation. The strength of *in vitro* suppressive effect may differ from that of $IL\text{-}10^+$ sorted cells due to the fact that not all $CD73^-CD25^+CD71^+$ produce IL-10 upon activation. Control populations ($CD25^-CD71^-$ or $CD73^+$ cells) were not suppressive at these ratios. A slight blocking effect in the $CD73^-CD25^+CD71^+$ Breg-mediated suppression was observed in the presence of anti-IL-10R mAb compared to

isotype control, suggesting a partial involvement of IL-10 in the suppression. This effect was however small and not statistically significant. It has to be noted that the $CD73^{-}CD25^{+}CD71^{+}$ B cell population does not include all of the $IL\text{-}10^{+}$ B cells. Therefore, $CD73^{-}CD25^{+}CD71^{+}$ expression can be used to identify a distinct population of Breg cells that does not include all of IL-10-producing B cells. Furthermore not all $CD73^{-}CD25^{+}CD71^{+}$ B cells produce IL-10. It is therefore possible that $CD73^{-}CD25^{+}CD71^{+}$ B cells may apply additional mechanisms to suppress T cell proliferation. The identification of the mechanisms involved will require significant research effort, as there can be many factors that may play a suppressive role besides IL-10. For example, the surface markers that were used to sort the cells may play a role similar to the suppressive role for CD25 by means of IL-2 deprivation that has been described in Treg cells.[198] In addition, blocking of CD80 ad CD86 has been shown to reverse Breg-mediated suppression of T cell responses.[159] Furthermore, these cells express PD-L1 and this interaction may also play a role as demonstrated in Treg cells.[101] Together, these data demonstrate that Br1 cells are highly potent suppressors of T cell responses. This is especially striking when compared to Tr1 and Treg cells, which suppress around 50% of antigen-specific proliferation at a ratio of one suppressor cell to two or four responder cells respectively.[101, 199]

In the experiments investigating the link of Br1 cells to immunoglobulin production we found that IgG4 production is specifically confined to $IL\text{-}10^{+}$ Br1 cells. TLR9 stimulation induced production of IgG1, IgG4 and IgA primarily in $CD27^{+}$ B cells while simultaneous stimulation with TLR9 and IL-10 induced specific upregulation of IgG4 particularly in $CD27^{-}$ B cells without affecting IgG1 and IgA. These findings combined with the fact that TLR9 stimulation induces IL-10 in a subset of B cells raised the question whether $IL\text{-}10^{+}$ cells are more prone to produce IgG4 than $IL\text{-}10^{-}$ B cells. There was approximately 10-fold increased IgG4 mRNA in $IL\text{-}10^{+}$ Br1 cells, compared to $IL\text{-}10^{-}$ counterparts. And when sorting Br1 cells from $CD27^{-}$ and $CD27^{+}$ subsets it emerged that IgG4 production was specifically upregulated in $CD27^{-}IL\text{-}10^{+}$ B cells suggesting that naïve Br1 cells are the main precursors of IgG4 producing cells.

Induction of allergen-specific IgG4 is a hallmark of successful peripheral tolerance induction as observed in allergen-SIT. Our data demonstrate that PLA-specific B cells from beekeepers mainly express IgG4. PLA-specific and non-PLA-specific B cells were isolated from peripheral blood and directly analyzed without *in vitro* culture. The elevated IgG4 expression in PLA-specific B cells indicates that these cells mainly represent circulating IgG4-switched PLA-specific memory B cells. Interestingly, IL-10 mRNA expression was also significantly higher in these cells suggesting that *in vivo* circulating PLA-specific B cells in beekeepers have elevated IL-10 production. Furthermore, when stimulated *in vitro* with TLR9-L, PLA-specific cells showed higher IL-10 secretion than non-PLA-specific B cells. This increased frequency of IL-10^+ cells among PLA-specific B cells was not observed in bee venom allergic patients. However after SIT the frequency of PLA-specific IL-10^+ B cells significantly increased to the same level as in beekeepers. PLA-specific IgG4 was detected at high concentrations in serum of non-allergic beekeepers who showed >1000 times lower PLA-specific IgE:IgG4 ratio than allergic individuals. Allergen-SIT and high-dose allergen tolerance has been linked to increased serum IgG4 and IL-10 production from T cells.[176] Here we demonstrate that allergen-specific B cell IL-10 production is increased during allergen-SIT. Our data demonstrate that particularly $CD27^-$IL-10^+ B cells are precursors of IgG4 producing cells. In addition the ongoing immune response of memory B cells contributes to IgG4. We found elevated IgG4 expression and an increased frequency of IL-10^+ cells among PLA-specific B cells in bee venom tolerant individuals. This suggests that in these individuals there exists a PLA-specific IgG4-switched memory B cell compartment that retains high IL-10 expression and may play a role in maintenance of tolerance. Whether these cells derive from Br1 cells requires further investigation. Interestingly, *Schistosoma mansoni* infection in children correlates with increased IL-10 production in response to parasitic antigens and decreased atopy.[200] Furthermore *Schistosoma mansoni* infection mediates protection against experimentally induced anaphylaxis. This protective effect was dependent on IL-10-producing B cells.[154] And, in line with our findings, high levels of IgG4 antibodies are detected in helminth-infected individuals.[201-203]

In conclusion, the current findings demonstrate an essential role for B cells in inducing and maintenance of immunological tolerance. The present study demonstrates two ways to directly purify and analyze these cells in humans according to IL-10 secretion and surface CD25, CD71 high and CD73 low expression. By analyzing CD27, it was observed that both naïve and memory subsets of Br1 cells can express IL-10 upon TLR9-L stimulation. Br1 cells act on T cell responses by interfering with $CD4^+$ T cell activation and they contribute to peripheral tolerance through production of non-inflammatory IgG4 antibodies. We demonstrated significant *in vivo* regulation of these cells by isolating major allergen-specific Br1 cells in high-dose allergen exposed beekeepers as well as *in vivo* induction of these cells after allergen-SIT of bee venom allergic subjects. Therefore, Br1 cells bring together two important arms of immune tolerance, namely play a role as suppressive cells and direct the humoral response towards IgG4, which together characterize a healthy immune response to allergens.

Acknowledgements

This work was supported by Swiss National Science Foundation (grant No. 320030-125249/1, 32-188226, 320030-140772), Christine Kühne-Center for Allergy Research and Education (CK-CARE) and the European Commission's Seventh Framework Programme (grant agreement No. 261357 (MEDALL).

Online repository figures

The online repository tables E1-E4 exceed the size suitable for printing and can be obtained from the original publication.[116]

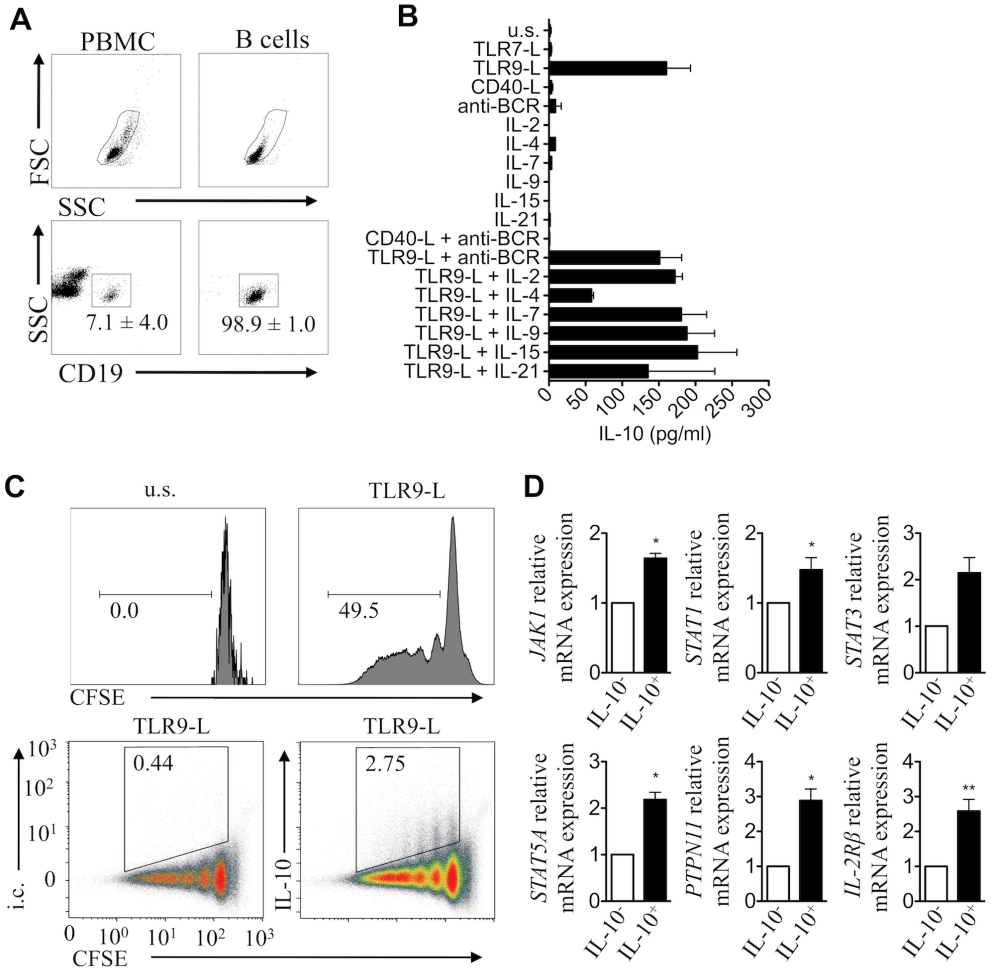
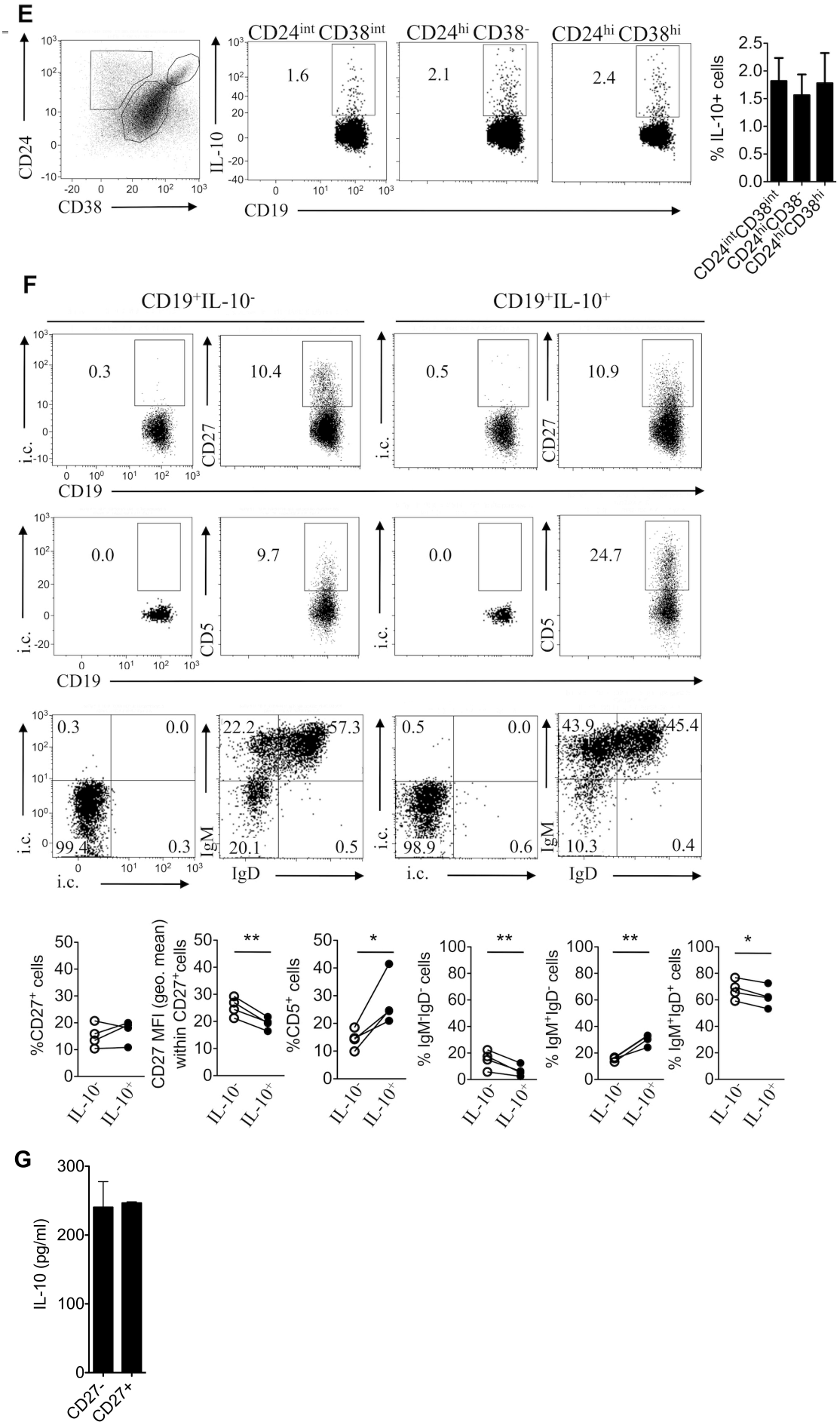


Figure E1. Isolation and characterization of IL-10$^+$ B cells. (A) Purification of human peripheral CD19$^+$ B cells. (B) IL-10 secretion by purified human peripheral B cells after 3 d stimulation with different B cell activators and common gamma chain cytokines. (C) CFSE dilution and IL-10 secretion in purified B cells. CFSE-labeled cells were left unstimulated or stimulated with TLR9-L for 5 d and analysed by flow cytometry. (D) Real-time PCR analysis of JAK1, STAT1, STAT3, STAT5A, PTPN11 and IL-2Rβ mRNA expression in IL-10$^-$ and IL-10$^+$ B cells measured directly after sorting. Expression is shown relative to IL-10$^-$ cells. (E) Frequencies of IL-10-secreting cells among CD24^{int}CD38^{int}, CD24^{hi} CD38$^-$ and CD24^{hi} CD38^{hi} B cells. Data are from 3 independent experiments. (F) Surface expression of CD27, CD5, IgM and IgD on IL-10$^-$ and IL-10$^+$ B cells. One representative example out of four independent experiments is shown. MFI refers to geometric mean fluorescence intensity. (G) IL-10 production by CD27$^-$ and CD27$^+$ B cells (n=3) after 3 d TLR9-L stimulation.


E
CD24
CD38
IL-10
CD19
CD24int CD38int
CD24hi CD38-
CD24hi CD38hi
1.6
2.1
2.4
% IL-10+ cells
CD24intCD38int
CD24hiCD38-
CD24hiCD38hi
F
CD19+IL-10-
CD19+IL-10+
i.c.
CD27
CD5
IgM
IgD
0.3
10.4
0.5
10.9
0.0
9.7
0.0
24.7
22.2
57.3
0.5
0.0
43.9
45.4
99.4
0.3
20.1
0.5
98.9
0.6
10.3
0.4
%CD27+ cells
CD27 MFI (geo. mean) within CD27+cells
%CD5+ cells
% IgM-IgD- cells
% IgM+IgD- cells
% IgM+IgD+ cells
IL-10-
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IL-10 (pg/ml)
CD27-
CD27+

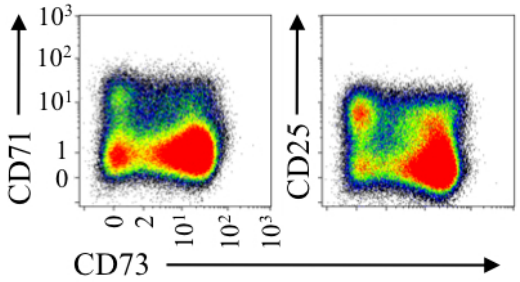

Figure E2. Flow cytometric analysis of surface expression of CD73, CD25 and CD71 on resting peripheral B cells. B cells were pre-enriched from PBMC and stained for CD19, CD73 CD25, CD71 and CD73. CD19$^+$ cells were gated and CD73 expression was plotted against CD25 and CD71 expression. One representative example out of 3 is shown.

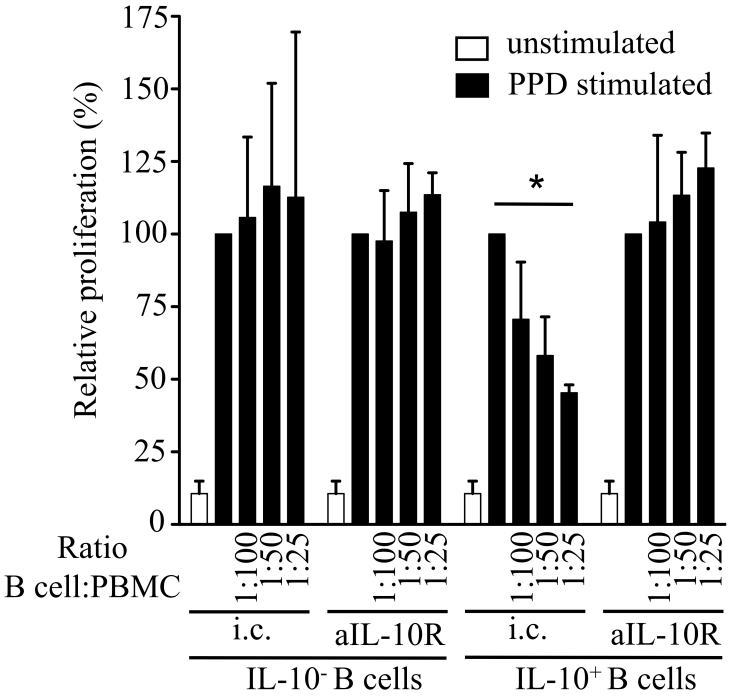

*Figure E3. IL-10$^+$ B cells suppress antigen-specific PBMC proliferation. [^{3}H]Thymidine incorporation measured in PBMC stimulated with PPD for 5 d with or without IL-10$^+$ or IL-10$^-$ B cells. Proliferation is shown relative to PPD stimulated PBMC. Data are from 3 independent experiments (n=6). *P<0.05.*

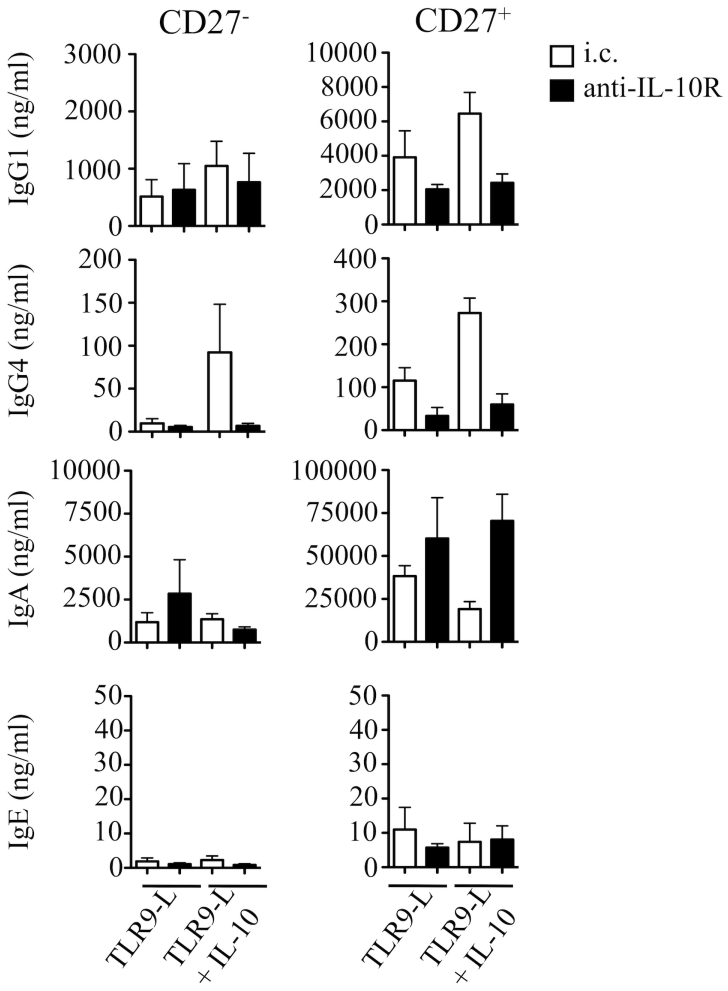

Figure E4. Effect of IL-10 neutralization on immunoglobulin production. CD27$^-$ and CD27$^+$ B cells were sorted by flow cytometry and cultured with TLR9-L or TLR9-L combined with IL-10 in the presence of blocking anti-IL-10R or isotype control (i.c.) antibodies. Secreted immunoglobulins were measured after 10 d.

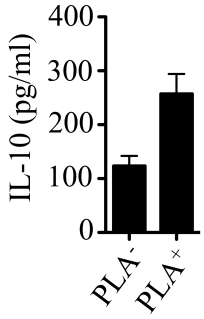

Figure E5. IL-10 production by PLA-specific B cells from beekeepers. IL-10 measured in supernatants from cultured PLA$^-$ and PLA$^+$ B cells. Sorted cells (2x10^4) were cultured for 3 d (n=2).

6.2 Human IL-10-overexpressing B cells possess extensive regulatory capacity toward both innate and adaptive immune responses

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Short title IL-10-overexpressing B cells exibit complex immunoregulatory phenotype

Key words IL-10 overexpression, regulatory B cells (Br1), suppressive capacity

Abstract

Background: Distinct human IL-10-producing B subsets with immunoregulatory properties have been described. However, the broader spectrum of cellular targets and mechanisms of suppression have not yet been extensively reported, particularly in respect of direct and indirect solely IL-10 mediated actions. Aim of the study was to investigate the effect of IL-10 overexpression in B cells on their phenotype and immunosuppressive capacity toward TLR-induced production of proinflammatory cytokines, maturation of dendritic cells, and antigen specific proliferation.

Methods: Purified B cells were transfected with pORF-hIL-10 and characterized in their profile of cytokine and immunoglobulin production (by ELISA and bead-based suspension assays), antigen presentation and co-stimulation capacity, transcription factors signature (by quantitative PCR and flow cytometry). Effects of autologous IL-10-overexpresing B cells on PBMC and monocyte-derived dendritic cells (MDDC) were addressed in co-cultures under stimulatory conditions.

Results: IL-10 overexpressing human B cells quickly acquired an immunoregulatory profile comprising upregulation of glycoprotein A repetitions predominant (GARP) and interleukin-2 receptor alpha chain (IL2RA). IL-10 overexpression in B cells was associated with a significant reduction in proinflammatory cytokine production (TNF-α, IL-8 and MIP-1α) and augmented secretion of anti-inflammatory IL-1Ra and VEGF. IL-10 overexpression was associated with a decrease in co-stimulatory potential. Co-cultures of autologous PBMC with IL-10-overexpressing B cells potently inhibited antigen-specific proliferation of PBMC and secretion of proinflammatory cytokines induced by TLR2 and TLR4 stimulation. Furthermore IL-10-overexpressing B cells suppressed MDDC maturation and expression of CD80, CD86 and CD83, while inducing expression of PD-L1.

Conclusions: Our data demonstrate a prominent role for IL-10 in inducing an immunoregulatory phenotype of B cells capable to exert substantial anti-inflammatory functions and contribute to modulation of immune responses providing a tolerance-inducing environment.

INTRODUCTION

B lymphocytes display a unique role in immunity through the production of antibodies. Nevertheless, B cells also substantially contribute to immune responses through antigen presentation and co-stimulation, cytokine secretion and lymphoid tissue organization.[169] B cells play an important role in the development of pathological immune responses such as asthma and atopic diseases (chronic immune reactivity to non-harmful antigens in sensitized individuals primarily mediated through IgE), autoimmunity (lack of control/excessive response to self antigens) and anti-tumor immunity/defense (insufficient response).[169, 171]

Since the late 1990s a growing body of evidence has accumulated supporting an immunoregulatory function for B cells. Distinct B cell subsets with suppressive potential were discovered in different models of infection,[154, 179] allergic inflammation,[156, 181] autoimmunity[137, 146, 177, 178] and cancer [180] and their actions were mainly linked with the provision of IL-10. Concomitant suppression of IgE and induction of IgG4 production as well as demonstration of strong antigen-specific IL-10 induction was observed in allergic patients that underwent specific immunotherapy (SIT) with successful outcome.[65, 102] These findings have raised interest in the involvement of regulatory B cells in the modulation of allergic diseases.[101, 171, 176] The existence of regulatory B cells in humans was supported by the finding that B cell depletion therapy in patients with rheumatoid arthritis and systemic lupus erithematosus (SLE) was associated with colitis exacerbations and induction of psoriasis.[166, 167] The best-described human regulatory B cell subsets are: I) $CD24^{hi}CD38^{hi}$ cells, which have been demonstrated to suppress the differentiation of T helper 1 cells in healthy individuals after stimulation with CD40L, but were functionally impaired in SLE patients[159]; II) B10 and B10pro cells were predominantly found within $CD24^{hi}CD27^{+}$ B cells that were able to negatively regulate monocyte cytokine production *in vitro*; III) TLR9-L induced IL-10^{+} Br1 cells were enriched among $CD25^{+}CD71^{+}CD73^{-}$ B cells and showed potent suppression of PPD-induced CD4+ proliferation.[161] These Br1 cells comprise two temporally distinct but spatially linked immunosuppressive functions: IL-10

production and IgG4 secretion that were demonstrated to be hallmarks of high-dose antigen-induced desensitization in bee venom allergic patients underwent successful specific immunotherapy. The link between PLA-specific B cells secreting IL-10 and undergoing preferential CSR toward IgG4 was confirmed in beekeepers as human *in vivo* tolerance model upon high-dose antigen exposure.[204] These findings particularly confirm the role of B cells in the maintenance/establishment of specific peripheral tolerance and their contribution to immune homeostasis.[171, 176] The suppressive mechanisms of most of the described regulatory B cell subsets are at least in part IL-10-dependent.

IL-10 is a pivotal anti-inflammatory cytokine not only protecting the host from excessive tissue damage during host defense reactions to pathogens, but also one of the key molecules critically involved in maintenance of immunological tolerance and homoestasis.[62, 205] IL-10 deficiency leads to development of spontaneous colitis.[131] IL-10 suppresses production of pro-inflammatory cytokines and chemokines as well as antigen presentation.[62] On the other hand, IL-10 promotes survival, proliferation, differentiation and class switch recombination in human B cells.[62] IL-10 suppresses IL-4-induced IgE production and enhances IgG4 production.[63, 64] However, addressing the exclusive role of IL-10 in the immunoregulatory potential of IL-10-producing B cells is largely biased by different initial antigen-dependent or antigen-independent signals. These signals, along with IL-10 production may lead to concurrent induction of other factors that may alter intrinsic IL-10-mediated regulatory features.

We hypothesized that IL-10 expression in normal human B cells together with its consequent IL-10R dependent signaling on the same cells is sufficient for B cells to acquire a regulatory phenotype, which in conjunction with secreted IL-10 can exert a suppressive profile impacting different niches of immune responses. We aim to characterize the phenotype and function of IL-10-overexpressing B cells in terms of cytokine secretion, antigen presentation

and co-stimulation, antibody production and suppressive capacity on both innate and adaptive immune responses.

MATERIALS AND METHODS

Isolation of PBMCs and B cells

Human PBMCs were isolated from heparinized peripheral venous blood or buffy coats from healthy adult volunteers using density gradient centrifugation on Ficoll (Biochrom, Berlin, Germany). Cells were then washed and resuspended in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% of heat-inactivated fetal calf serum and antibiotics (penicillin, streptomycin, kanamycin), MEM vitamin, L-glutamine, nonessential amino acids and sodium pyruvate (Life Technologies), further on in the text referred as complete RPMI medium or culture medium. B cells were purified from PBMC by negative selection. Non-B cells were labeled with a cocktail of biotin-coupled antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a, and anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by magnetic separation (AutoMACS, Miltenyi Biotec). The purity of isolated $CD19^+$ B cells was assessed by flow cytometry using anti-CD19 antibody and routinely resulted in >96% of $CD19^+$ cells. For some experiments pre-enriched B cells were labeled with anti-CD27 antibody and $CD27^-$ (primarily naive) and $CD27^+$ (primarily memory) B cells were sorted using FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ USA).

Plasmids

An expression plasmid containing the IL-10 gene construct - pORF-hIL-10 and its backbone containing counterpart pORF-mcs (both from Invitrogen, Life technologies) were introduced in DH5α strain of electrocompetent bacteria and propagated overnight under ampicilin-selection at 37° C. Plasmid DNA MaxiPreps EF kits (Qiagen) were used for purification of plasmid DNA. Their concentration was determined using NanoDrop 2000 and adjusted to 1 mg/ml.

Transfection of B cells

Freshly purified B cells were transfected with either pORF-hIL-10 (IL-10_trB) or pORF-mcs (ctrl_trB) plasmid vectors using nucleofection (Human B cell nucleofection kit and Nucleofector, Amaxa, Lonza Cologne AG, Germany). Then, cells were washed, centrifuged at 250xg/8 min, resuspended in complete RPMI and used for experiments. For the optimization of the transfection protocol B cells were transfected with a commercial pmaxGFP construct (Lonza) and transfection efficiency along with cell death exclusion (7-AAD) was assessed by flow cytometry 24h post-transfection. For some experiments B cells were stimulated with 1 μM of synthetic phosphorothioate B type TLR9-L CpG 2006 (Microsynth GmbH, Balgach, Switzerland) for 72h prior to transfection.

Preparation of monocyte-derived dendritic cells (MDDCs)

PBMCs were seeded in at a density of $2.5x10^6/cm^2$. Monocytes were obtained as a fraction of adherent PBMC after 2 h while the rest of the cells were washed away with pre-warmed complete RPMI medium. A combination of 10 ng/ml GM-CSF (PeproTech, London, UK) and 50 ng/ml IL-4 (Novartis) was used to induce differentiation of monocytes to dendritic cells (MDDCs) over the period of 7 days. Differentiation was confirmed by down-regulation of CD14 expression and retained/up-regulated expression of CD11c.

Cell cultures and co-cultures

All B cell culture conditions were set in a format of $1x10^6$ cells/ml in complete RPMI. Cell culture supernatants were collected for secreted cytokine quantification and cell lysates for mRNA expression analysis after 24h. Furthermore, cells were harvested for surface and intracellular proteins expression measurement by flow cytometry. For the co-culture studies B cells were stimulated prior to transfection with 1 μM of synthetic phosphorothioate B type TLR9-L CpG 2006 (Microsynth GmbH, Balgach, Switzerland) for 72h.

In order to test the effect of IL-10-overexpressing B cells on TLR-Ls induced cytokine secretion from PBMC, maturation of MDDC and antigen specific

proliferation of PBMC freshly isolated and IL-10 transfected autologous B cells were co-cultured at different cell number ratios in respect to the target cells which were kept at fixed density of $1x10^6$ target cells/ml in complete RPMI. Cells were left rested in wells for 2h before appropriate stimuli were added. For the maturation of MDDCs, co-cultured cells were stimulated with 50 ng/ml TLR4-L lipopolysaccharide (LPS) from E.coli 0111:B4 (Sigma-Aldrich, St. Louis, MO USA) for 36h. Cell culture supernatants were collected for secreted cytokine quantification and cells harvested for surface marker expression analysis after 24h and 36h, respectively. For the induction of cytokine secretion from PBMC, co-cultures were incubated in complete medium with addition of either 100 ng/ml TLR2-L lipoteichoic acid from S. aureus (LTA-SA, Invivogen) or 50 ng/ml of TLR4-L LPS for 24h. Supernatants were taken after 24h for quantitative cytokine measurement. Antigen specific stimulation of PBMC in co-cultures was elicited by tetanus toxoid antigen (TT, Serum Institute) during 5 days. Cells were then pulsed with 1 μCi of [^{3}H]-thymidine/well (DuPont; New England Nuclear), and the incorporation of labeled nucleotide was measured in an LKB β plate reader (GE Healthcare).

Flow cytometry

After isolation or cell culture cells were harvested, centrifuged at 300xg for 8 min at 4° and resuspended in ice-cold PBS. Cells were then incubated with fixable viability dyes eFluor450 or eFluor780 (eBioscience, San Diego, CA, USA) in PBS for 30 minutes on ice and then washed with staining buffer (0.5% BSA and 2 mM EDTA in PBS). For the expression of surface markers cells were stained with following antibodies: CD19-FITC, CD80-FITC, HLA-DR-ECD (Beckman Coulter, Fullerton, CA USA), IgD-FITC, IgM-PerCP/Cy5.5, CD27-PE, CD83-PC5, CD86-PE, CD274-FITC (BD Biosciences, Franklin Lakes, NJ USA), CD5-PC7, CD11c-PacBlue, CD14-PE-Cy7, CD19-APC/Cy7, CD19-BV510, CD24-BV421, CD27-FITC, CD38-PerCP/Cy5.5, CD38-PC7 (Biolegend, San Diego, CA USA), CD1d-PE (eBioscience) in staining buffer for 30 min on ice. Cells were washed once, resuspended in 0.5% PFA/PBS and measured. Corresponding isotype control antibodies were used as negative controls. Samples were analyzed using Galios flow cytometer

(Beckman Coulter) or sorted using FACSAria II (Becton Dickinson). Data were analyzed using Kaluza software (Beckman Coulter).

RNA isolation and reverse transcription

RNA was extracted from cell lysates using RNeasy kits (Qiagen, Hilden, Germany) modified to additionally include DNAse digestion step. Reverse transcription was performed with reverse-transcription reagents (Fermentas, St.Leon-Rot, Germany) including random hexamers according to manufacturer's recommendations.

Real-time PCR

cDNA samples were amplified using target gene specific primers (Microsynth) and SYBR green PCR master mix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's suggested protocol on ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA). ΔCt method was used to obtain relative quantification of gene transcription to housekeeping gene EF1α. Subsequently, comparative $\Delta\Delta$Ct method was performed to obtain relative quantification among different samples. The following primers pairs were designed according to sequences reported in GeneBank and used: AID: fw 5'-AGAGGCGTGACAGTGCTACA-3' rv 5'-TGTAGCGGAGGAAGAGCAAT-3'; BCL-6: fw 5'-AGACCGTCCATACCGG-3' rv 5'-CGCAAGTGAAGTCGCA-3'; BLIMP-1: fw 5'-AGCTGACAATGATGAATCTCA-3' rv 5'-GTGAAATGTTAGAACGGTAG AG-3'; CD40: fw 5'-TTGGGGTCAAGCAGATTGCTA-3' rv 5'-GCAGATGACACATTGGAGAAGA-3'; EF1a: fw 5'-CTGAACCATCCAGGCCA AAT-3' rv 5'-GCCGTGTGGCAATCCAAT-3'; GARP: fw 5'-GCCCTGTAAGAT GGTGGACAAG-3' rv 5'- CAGATAGATCAAGGGTCTCAGTGTCT-3'; IgA: fw 5'-CGCTGGCCTTCACACAGAA-3' rv 5'-CGCCATGACAACAGACACA-3'; IgE: fw 5'-ACACATCCACAGGCACCAAA-3' rv 5'-TTGCAGCAGCGGGTCAA-3'; IgG1: fw 5'-CTCTCAGCCAGGACCAAGGA-3' rv 5'-GGTGGGCATGTGT GAGTTTTG-3'; IgG4: fw 5'-ACC$^{C}/_{A}$TGGTCACCGTCTCCTCA-3' rv 5'-GGGACCATATTTGGACTC-3'; ICOS-L: fw 5'-CTCCGCCCGCACCAT-3' rv 5'-CTACCATCGCTCTGACTTCCTTCT-3'; IL-10: fw 5'-GTGATGCCCCAAGCTG

AGA-3’ rv 5’-CACGGCCTTGCTCTTGTTTT-3’; IL2RA: fw 5'-AAACTCTAGCCACTCGTCCTG-3' rv 5'-ACTTGTTTCGTTGTGTTCCGA-3'; IRF-4: fw 5'-AACGCCTTACCCTTCG-3' rv 5'-CCCGGTAGTACAGGCA-3'; PAX-5: fw 5'-CTGATCTCCCAGGCAAACAT-3' rv 5'-TTGCTCATCAAGGTG TCAGG-3'; PDL1/CD274: fw 5'-ACTGGGACATTCGGGTTTTGA-3' rv 5'-CCTCACTTTCTGAGCGATGAGT-3'; PDL2: fw 5'-GCATAATAAGATGGCTC CCAGAA-3' rv 5'-AAAGAGGGAAGTGAACAGTGCTATC-3'; SOCS-3: fw 5'-CTTCAGCATCTCTGTCGGAAGA-3' rv 5'-GCATCGTACTGGTCCAGGAACT-3'; XBP-1: fw 5'-AGGAGTTAAGACAGCGC-3' rv 5'-GAGTCAATACCGCCAG-3'.

Quantification of cytokines and immunoglobulin isotypes

Cell culture supernatants were quantified for IL-10 content using specific ELISAs. Other secreted cytokines and immunoglobulins were quantitatively determined using fluorescence-immuno-coupled beads-based multiplex assays: Bio-Plex Hu Cytokine panel, 17-Plex Group I or 27-Plex Group I or Bio-Plex Pro™ Human Isotyping Panel (all from Bio-Rad Laboratories) according to manufacturer’s instructions, respectively. Fluorescent signals were read and analyzed using the Bio-Plex 200 System (Bio-Rad Laboratories).

Statistical Analysis

Unless otherwise indicated presented data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 statistical tests. Wilcoxon signed rank tests and Paired *t*-tests were used for assessing statistical significance. Statistically significant scores are shown on the graphs indicating significantly changed values between samples having P values < 0.05.

RESULTS

Overexpression of IL-10 in human B cells

Plasmid mediated gene transfer in normal PBMC purified B cells was efficient using nucleofection. Nevertheless, it came with a certain cost in viability of the transfected cells. Transfection conditions were tested varying B cell number and different amounts of either pmaxGFP or pORF-hIL-10 or pORF-mcs (backbone control) vectors (Suppl Fig1A, 1B). The transfection protocol was optimized to use 2-5x10^6 B cells and 5-10 μg of DNA plasmid per transfection. It typically resulted in approximately 50% of GFP+ cells transfected (GFP+ cells) among approximately 75% of viable cells, as well as in significant and plasmid amount dependent-IL-10 release after 24h (Suppl Fig1B).

IL-10-overexpressing B cells potently suppress production of proinflammatory cytokines while remarkably enhancing release of IL-1Ra and VEGF

An average of approximately 100-fold increase in secreted IL-10 was found in cell culture supernatants (Figure 1A) and approximately 3500-fold increase in IL-10 gene transcripts in lysates of B cell transfected with pORF-hIL-10 (IL-10_tr) when compared with pORF-mcs (ctrl_tr) transfected samples 24h post-transfection. The transfection procedure did not significantly affect the stimulatory effect of TLR9-L on IL-10 induction compared to non-transfected B cells (non_tr) (Suppl Fig1C). High IL-10 production in IL-10-overexpressing B cells was accompanied by a significant decrease in production of proinflammatory TNF-α, IL-8 and MIP-1α, while release of anti-inflammatory IL-1Ra and VEGF was significantly enhanced in comparison to control cells (Figure 1B). Among the other cytokines detected there was a tendency for IP-10 and MIP-1β to be inhibited, while detectable levels of IL-6, IFN-γ, RANTES, G-CSF, GM-CSF, FGF and PDGF did not significantly change.

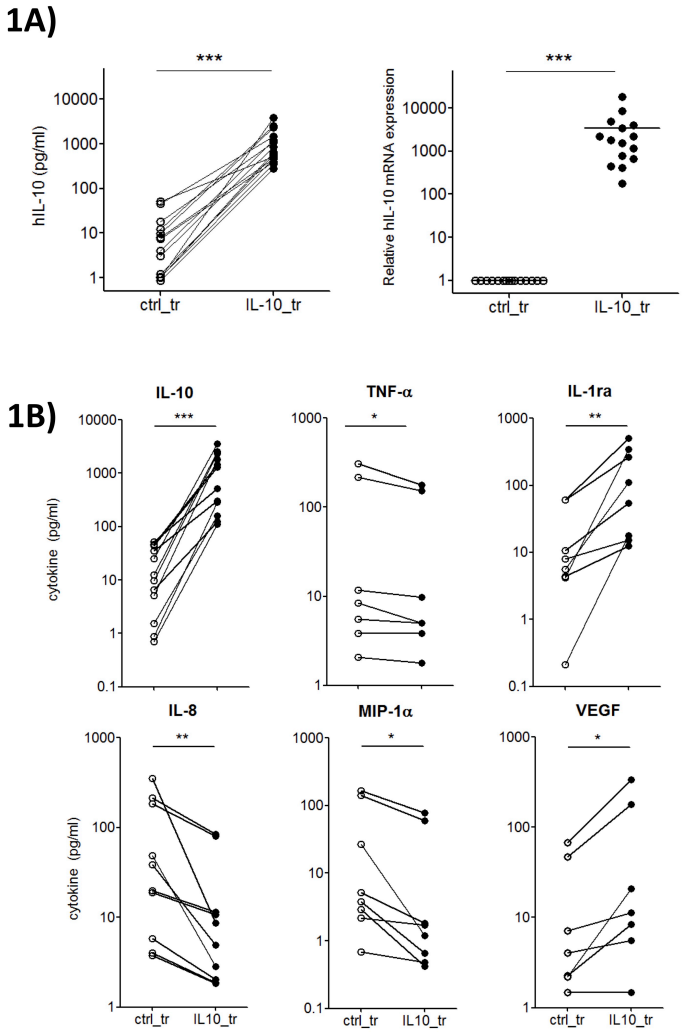


Figure 1. Overexpression of human IL-10 and profile of concurrent cytokine release from IL-10-overexpressing B cells. *B cells were transfected with pORF-hIL-10 (IL10_trB) or pORF-mcs (ctrl_trB) and cultured in medium for 24h. (**A**) Secreted human IL-10 and relative human IL-10 mRNA expression were measured in cell culture supernatants or cell lysates using specific ELISA or beads-based multiplex cytokine measurement, and quantitative-PCR, respectively (n=15 donors). (**B**) Secretion of other cytokines, chemokines and growth factors was quantified using beads-based multiplex cytokine measurement (n=12). Statistical analysis of the results was performed using Wilcoxon matched-pairs signed rank test and significant changes indicated as *p<0.05, **p<0.01,***p<0.001.*

IL-10-overexpressing B cells acquire a complex immunoregulatory phenotype comprising upregulated expression of GARP, IL-2RA, PD-L1 and CD38

IL-10-overexpressing B cells were characterized in terms of their gene expression on mRNA (24h) and protein level (36h) relative to control vector transfected B cells. There was a significant increase in expression of the directly IL-10-responsive suppressor of cytokine signaling-3 (SOCS3) gene in IL-10-overexpressing B cells (Figure 2A). A similar tendency for SOCS1 gene was observed (data not shown). At the same time, there was no significant effect on the transcription of IL-10RA chain (data not shown) suggesting no direct negative feedback mechanism on expression of the IL-10 receptor component. Among molecules previously described to be linked with the function of regulatory T cells: glycoprotein A repetitions predominant (GARP) and interleukin-2 receptor α chain (IL-2RA/CD25) were significantly upregulated in IL-10-overexpressing B cells. No difference was observed on the transcriptional level of TGF-β (data not shown). We also measured the expression of surface molecules that have been linked with different murine and human regulatory B cells. Expression of CD38 was significantly higher, with the same tendency observed for CD5 in IL-10-overexpressing human B cells while the expression of CD1d and CD24 was not changed (Figure 2A).

Expression of different co-stimulatory molecules, HLA-DR, a MHC class II molecule responsible for the presentation of processed antigens to CD4+ T cells, and CD40 were assessed in IL-10-overexpressing B cells in order to address their role in antigen presentation and co-stimulation. Along with high IL-10 expression there was a significant decrease in the expression of CD80 and a trend in the same direction for CD86 expression, while there was no change in expression of HLA-DR and CD83 (Figure 2B). In addition, expression of PD-L1, OX40-L, and CD40 was also significantly elevated at mRNA level. Expression of PD-L2 and ICOS-L showed no difference (Figure 2B).

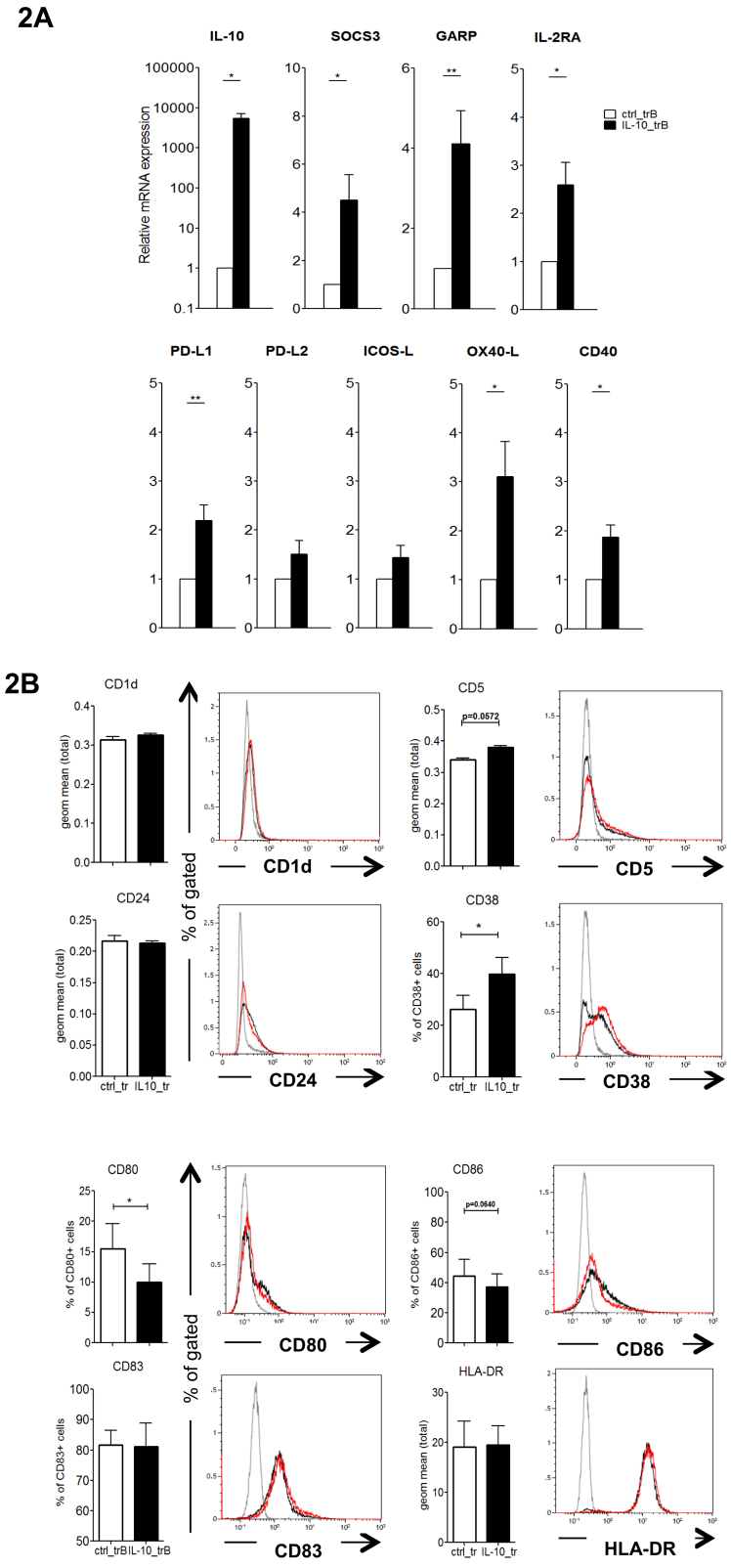


Figure 2. Expression profile of immunosuppression- and costimulation-related genes in IL-10-overexpressing B cells. ***(A)*** *mRNA Expression of immunoregulatory molecules and members of B7 family, and* ***(B)*** *expression of surface molecules involved in immunoregulation, costimulatory molecules and HLA-DR by flow cytometry, 24h and 36h post-transfection, respectively (n=4-9). Statistical analysis was done according by Paired t-test *p<0.05, **p<0.01.*

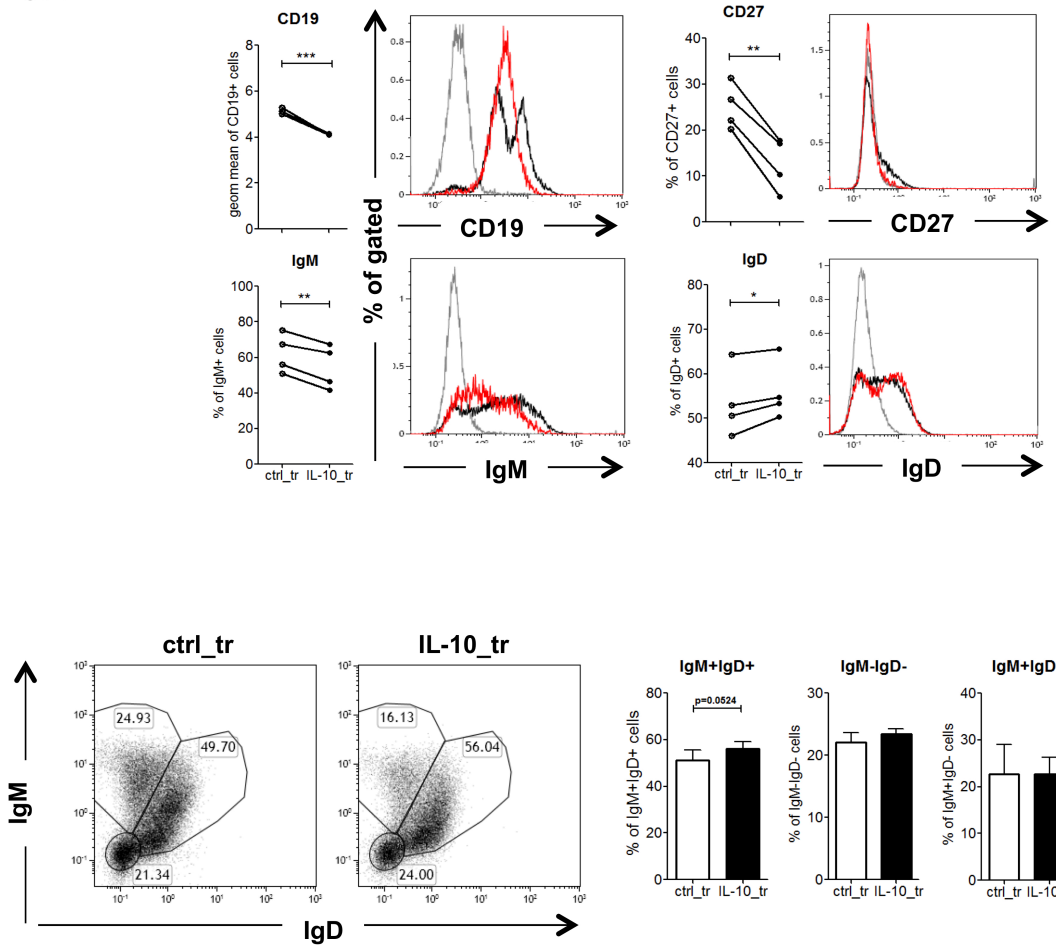
Profile of IL-10–overexpressing cells is skewed toward partially activated plasmablasts

In order to assess the activation status of IL-10-overexpressing B cells analysis of distinct surface markers and surface immunoglobulins from was performed. Overexpression of IL-10 in B cells resulted in consistent and significantly lower expression of CD19, CD27 and total surface IgM, while an increase in IgD expression was observed 36h post-transfection. The percentage of IgM+IgD+ as well as IgM-IgD- B cells tended to increase while there was no change in frequency of IgM+IgD- upon IL-10 overexpression (Figure 3A). Transcription factor genes expression showed a profile with a significant increase in IRF-4 and XBP-1 but not BLIMP-1 as a consequence of IL-10 overexpression (Figure 3B).

Effect of IL-10 overexpression on class-switch recombination and isotype profile of immunoglobulin production

Transcripts encoding AID, an enzyme crucial for CRS, and IgA were significantly decreased in IL-10-overexpressing B cells 72h after transfection. The transcription level of IgE, IgG1 and IgG4 was not significantly altered compared to control cells (Figure 3C). In order to determine secretion of immunoglobulins B cells prestimulated for 3 days with TLR9-L, transfected to overexpress IL-10 and incubated in culture medium for 7 days. A trend for less IgE secreted was observed in IL-10-overexpressing B cells when compared with control vector transfected samples (Figure 3D). There was no difference in the amount of other isotypes secreted between two samples. In order to address potential differences in IgG4 and IgE production among IL-10-overexpressing naïve and memory B cells, naïve B (CD19+CD27-) and memory B (CD19+CD27+) cells were transfected to overexpress IL-10 and stimulated with TLR9-L for 10 days post-transfection. Among all isotypes measured there was the highest decrease in concentration of secreted IgE observed, particularly pronounced in memory B cells overexpressing IL-10 when compared with control transfected memory B cells (Figure 3E).

3A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IL-10_tr
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21.34
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24.00
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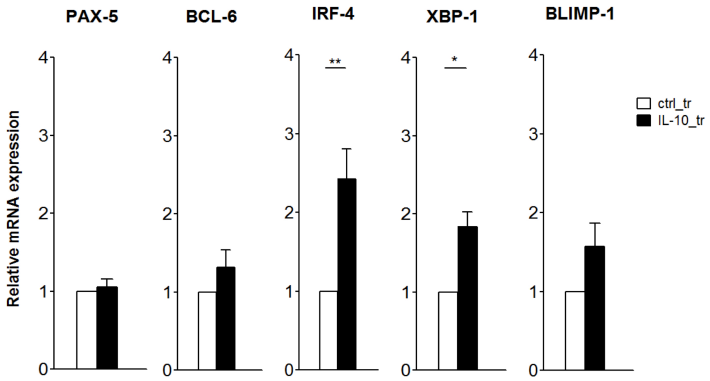
% of IgM+IgD- cells

3B


PAX-5
BCL-6
IRF-4
XBP-1
BLIMP-1
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*
Relative mRNA expression
ctrl_tr
IL-10_tr

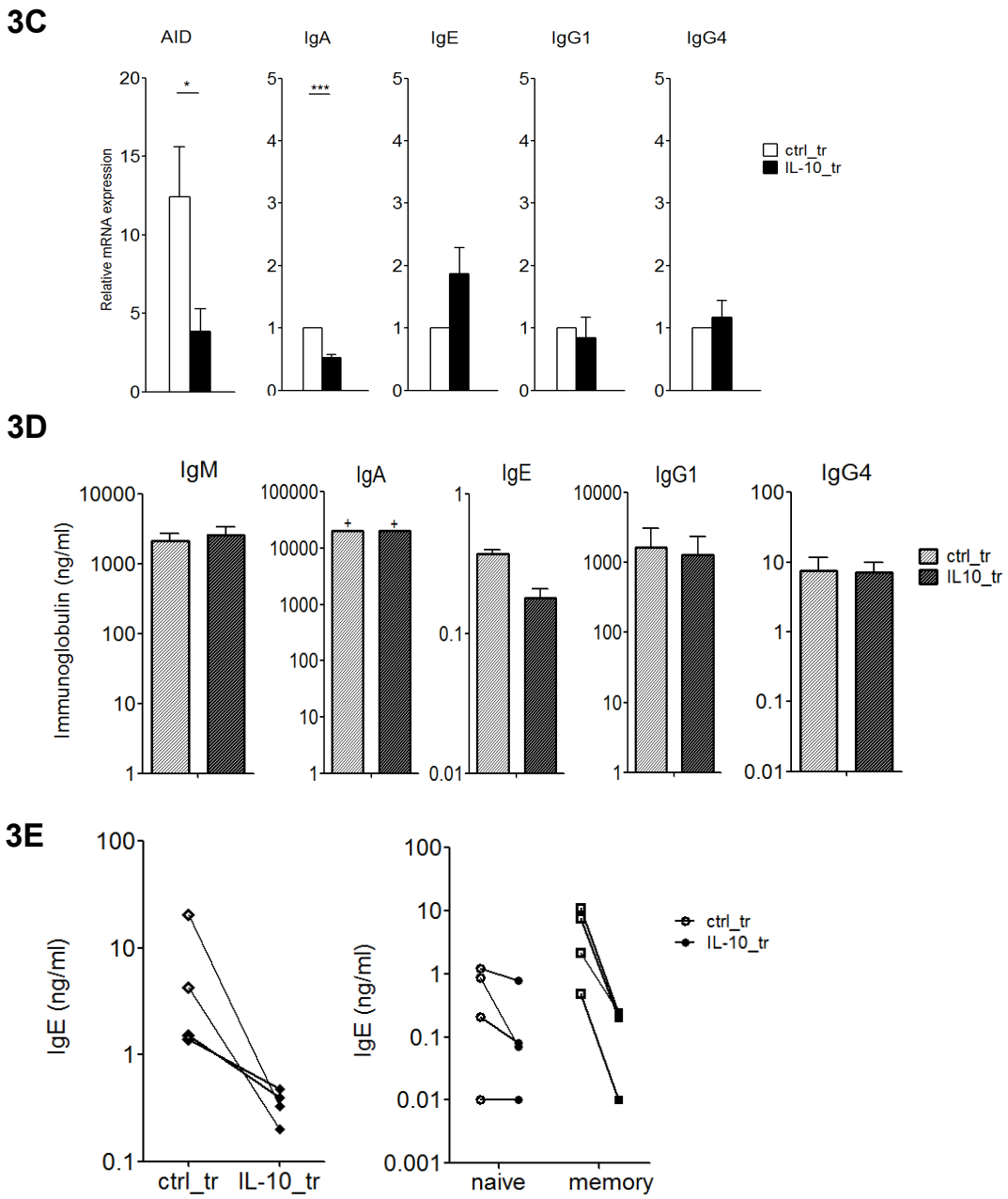


Figure 3. Surface activation phenotype, transcription facors signature and profile of immunoglobulin production in IL-10-overexpressing B cells. *Expression of (**A**) B cell activation-related molecules CD19, CD27 and surface immunoglobulins IgM and IgD was assesed by flow cytometry 36h post-transfection. mRNA expression of (**B**) transcription factors and (**C**) AID and immunoglobulin genes was determined 24h and 72h post-transfection, respectively using quantitative PCR (n=4-9). (**D**) B cells were preconditioned with TLR9-L for 72h, transfected and cultured in medium for additional 7days. Secreted immunoglobulins were measured using multiplex beads-based isotyping measurement (n=2). (**E**) Cell culture supernatants of either total B or sorted naive B (CD19+CD27) or memory B (CD19+CD27-) cells transfected with pORF-hIL-10 or pORF-mcs and subsequently stimulated with TLR9-L were quantified for secreted IgE 10 days post-transfection using the same method (n=4). Statistical analysis was done by paired t-test but no statistical significant results were found.*

IL-10–overexpressing B cells inhibit TLR2-L and TLR4-L-induced cytokine release from PBMC

In order to test the effect of IL-10–overexpressing B cells on TLR2-L and TLR4-L-induced cytokine production from PBMC, freshly purified B cells were pre-stimulated with TLR9-L (72h), transfected to overexpress IL-10, co-cultured with autologous PBMC in cell number ratio B:PBMC=1:4 and stimulated with either TLR2-L or TLR4-L during 24h. Both TLR2-L- and TLR4-L-induced production of proinflammatory cytokines including TNF-α, IL-1b, IL-6, IL-10, IFN-γ, IL-8, MCP-1, MIP-1β, G-CSF and GM-CSF in supernatants of PBMC cultures (Figure 4). However, there was an increase in cytokine release in control vector transfixed B cell co-cultures with no stimulus added, which might be due to a presence of small proportion of dead/dying cells after transfection. In IL-10-transfected B cell co-cultures the significant IL-10 overproduction was associated with substantial inhibition of proinflammatory TNF-α, IL-1b, IL-6, IL-8, IFN-γ and G-CSF induced on both TLR2- and TLR4-triggered pathways, when compared with control vector-transfected B cell co-cultures under both stimulatory and nonstimulatory conditions (55-98% of inhibition), also in comparison with stimulated PBMC samples (60-95% of inhibition). There was no significant change in the extent of inhibition between IL-10- and control-transfected B cell co-cultures on production of MCP-1 and MIP-1β induced by either TLR2-L or TLR4-L in comparison with stimulated PBMC alone.

IL-10-overexpressing B cells suppress the maturation of MDDCs and inhibit their cytokine release upon LPS stimulation

To test their effect on MDDC maturation, freshly isolated B cells were prestimulated with TLR9-L for 72h, transfected to overexpress IL-10 and subsequently co-cultured with autologous MDDC in B cell:MDDC ratios 1:2, 1:4 and 1:8. Maturation of MDDC was induced with LPS during 36h, which typically resulted in complete loss of CD14 expression with concomitant upregulation of HLA-DR, CD80, CD86, and CD83). The level of LPS-induced downregulation of CD14 expression on CD11c+ MDDCs was reduced in cultures of MDDCs with IL-10-overexpressing B cells compared to controls

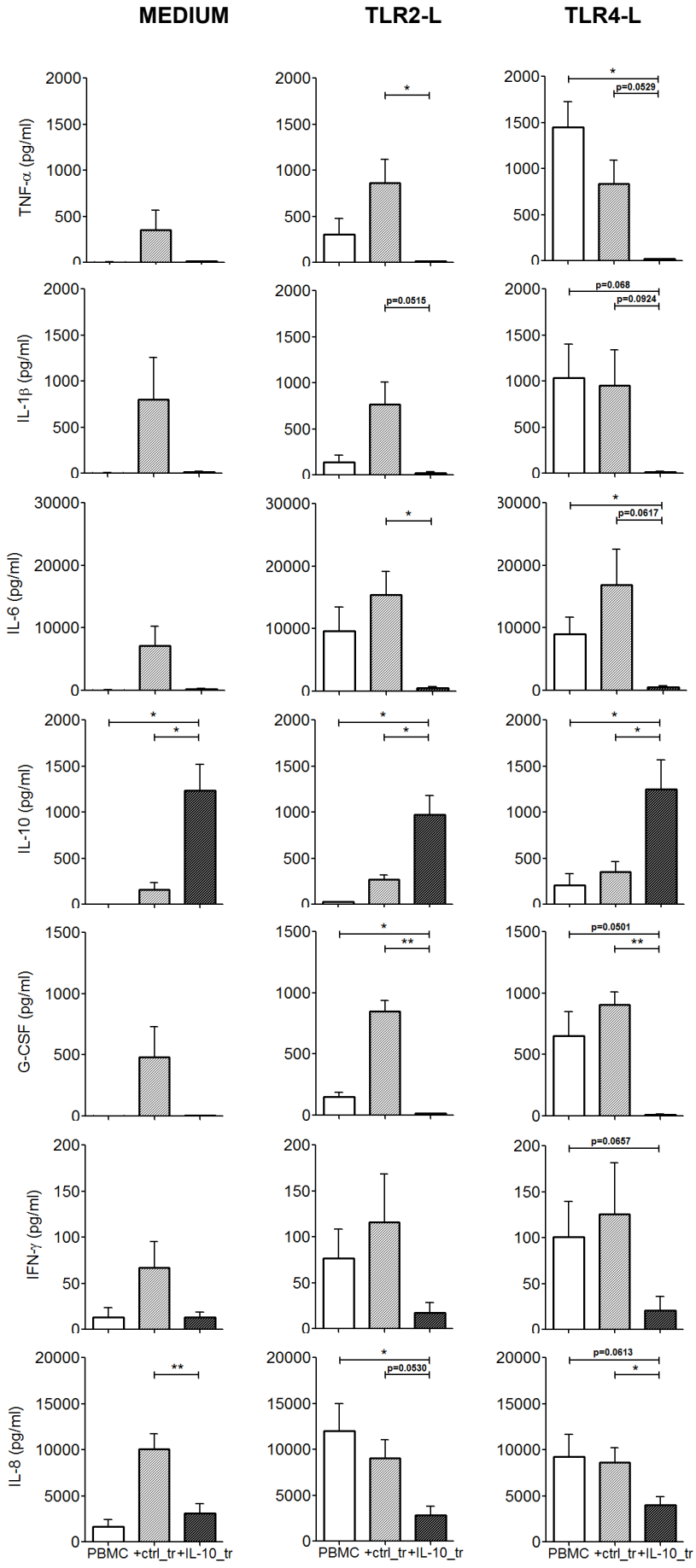
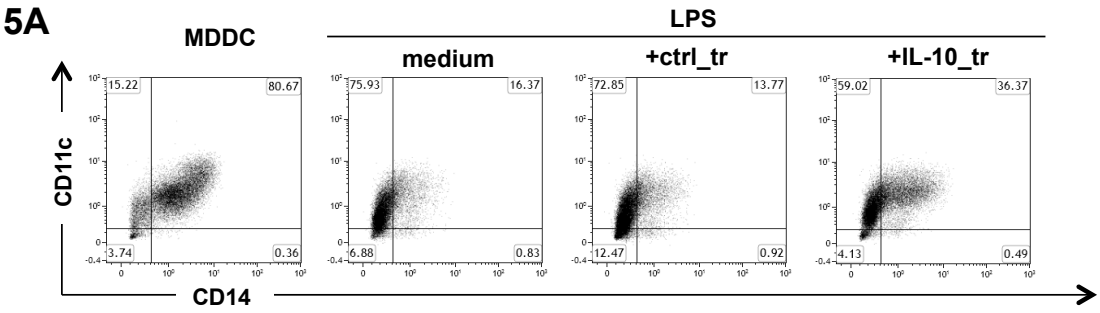


Figure 4. Cytokine secretion from PBMC co-incubated with TLR9-L pretreated and transfected B cells upon TLR2-L or TLR4-L stimulation. *Purified B cells were prestimulated with TLR9-L (72h), transfected either with pORF-hIL-10 or pORF-mcs, co-cultured with autologous PBMC (B:PBMC ratio= 1:4) and stimulated with TLR2-L or TLR4-L (or left nonstimulated). Secreted cytokines, growth factors and chemokines were quantified in cell co-culture supernatants using beads-based multiplex cytokine measurement 24h after stimulation (n=4). Statistical analysis was done by paired t-test (*p<0.05, **p<0.01).*

(Figure 5A). The profile of co-stimulatory molecules on IL-10-overexpressing B cells co-incubated MDDC included reduced expression of CD80, CD86 and CD83 but augmented expression of PD-L1 when compared with control transfected co-cultured samples (Figure 5B). Expression of HLA-DR on MDDC was not affected. Additionally, in the supernatants of MDDCs co-cultured with IL-10-overexpressing B cells proinflammatory TNF-α, IL-12p70, IFN-γ and G-CSF were inhibited, while at the same time IL-10, IL-1Ra and VEGF were elevated compared with control co-cultures (Figure 5C). All of these effects were dependent on the B cells:MDDC ratio. RANTES and MCP-1 were inhibited to a similar extent in both IL-10- and control vector-transfected B cell co-cultures only at the highest B cells ratio added. Levels of IL-6, IL-8 and MIP-1β exceeded the assay's upper detection limit.

IL-10-overexpressing B cells potently inhibit antigen-specific proliferation of PBMC

Freshly isolated B cells were transfected to express IL-10 or transfected with the control vector or left non-transfected and co-incubated with autologous PBMC (ratio 1:4) in the presence of TT antigen during 5 days. Co-incubated non-transfected B cell did not have a significant effect (22% of inhibition) while control transfected B cells significantly inhibited TT induced PBMC proliferation (56% of inhibition). IL-10-overexpressing B cell co-cultured with PBMC strongly suppress antigen specific proliferation (86% of inhibition) of autologous PBMC (Figure 6).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CD11c
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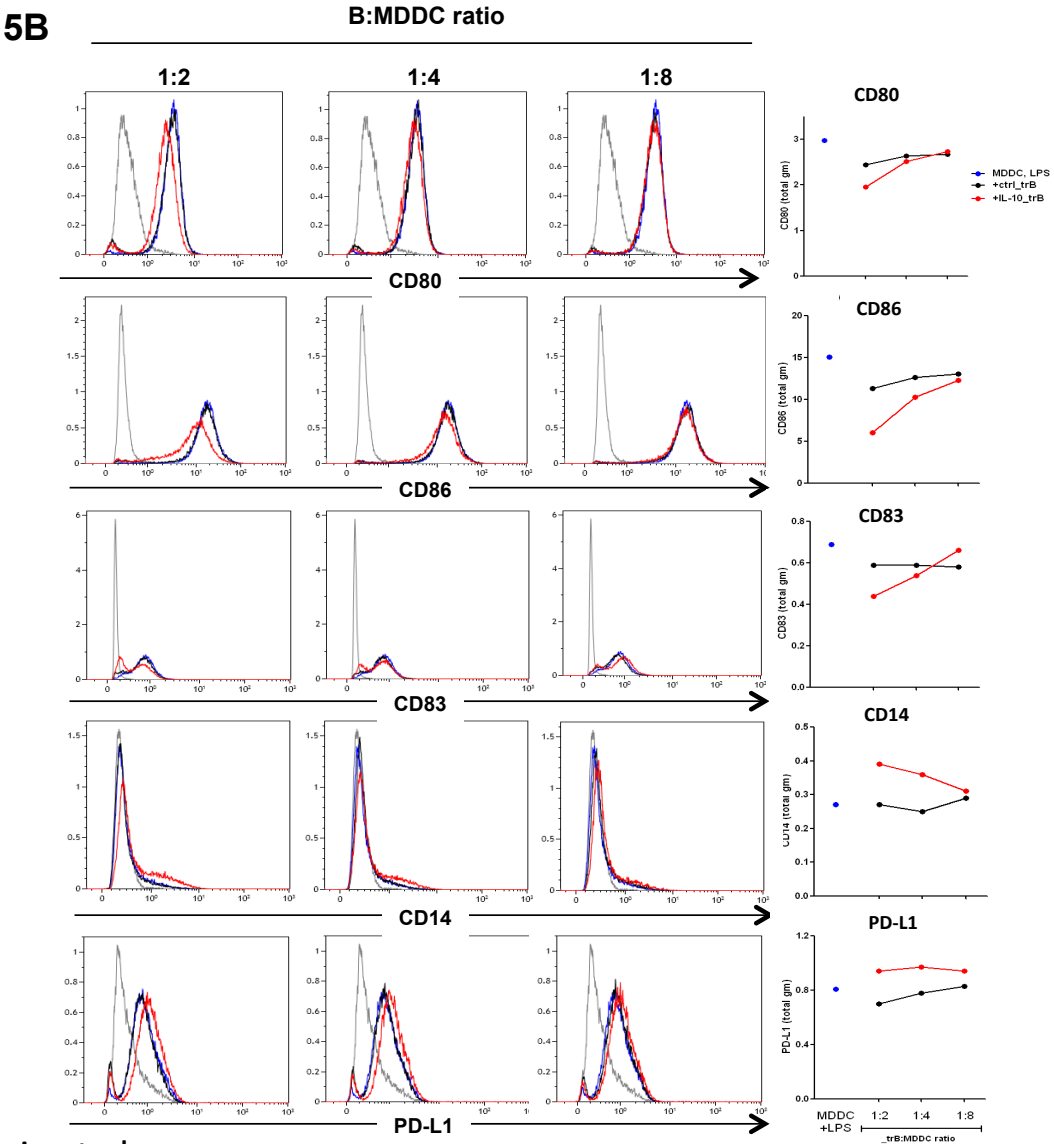
5B
B:MDDC ratio
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+IL-10_trB
MDDC
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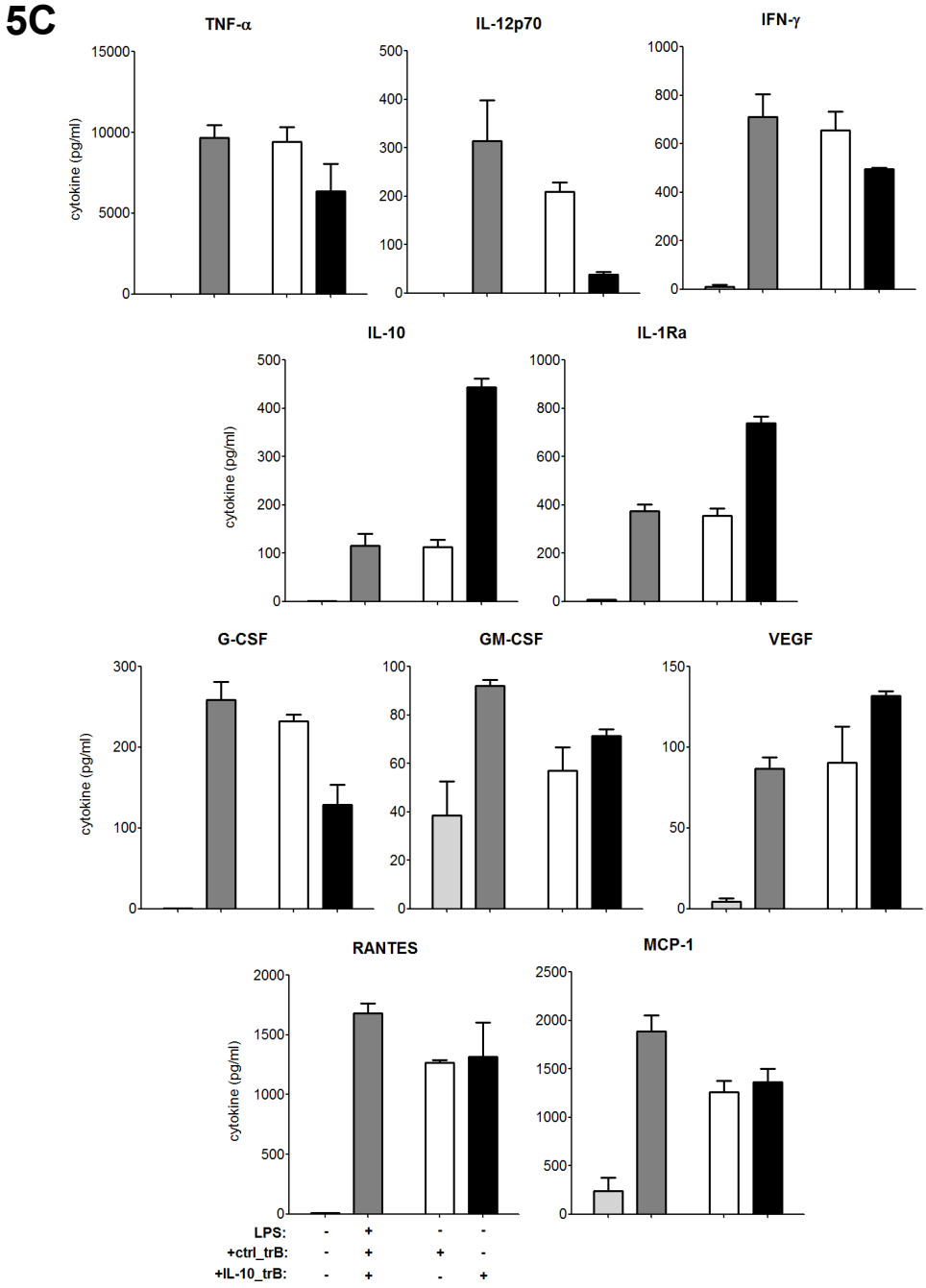


Figure 5. MDDC maturation, expression of co-stimulatory molecules and cytokine release after co-incubation with TLR9-L pre-activated and IL-10-transfected B cells upon stimulation with TLR4-L. *MDDCs were derived from plastic–adhered monocytes differentiatied with GM-CSF + IL-4 for 5 days. Autologous B cells were pretreated with TLR9-L for 72h, transfected with pORF-hIL-10 (hIL-10_trB) or pORF-mcs (ctrl_trB), co-incubated with MDDC (in three cell number ratios: 1:2. 1:4, 1:8) and subsequently stimulated with TLR4-L for 36h. (**A**) Expression of CD14 and CD11c and (**B**) and co-stimulatory molecules was assessed by flow cytometry. One donor (out of two) results is presented. (**C**) Cytokine secretion was quantified in supernatants using beads-based multiplex cytokine measurement (n=2).*

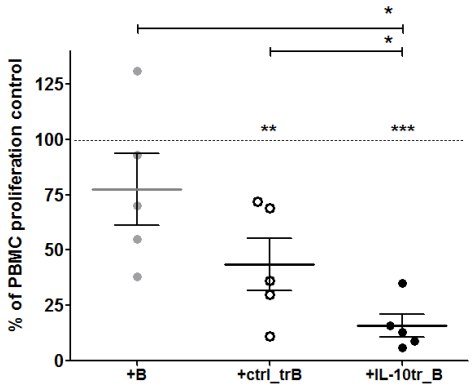


Figure 6. Antigen-specific proliferation of PBMC co-incubated with IL-10-overexpressing B cells. *B cells were transfected to overexpress IL-10 and co-cultured with PBMC following stimulation with tetanus toxoid (TT) for 5 days (B cell:PBMC=1:4). [^{3}H]-thymidine was added to cultures for last 8h of culture. Cells were harvested and proportion of incorporated thymidine quantified. Results are presented as proliferation relative to TT treated PBMC sample (proliferation control set as 100) and expressed in % (n=5). Statistical significance was performed using paired t-test (*p<0.05, **p<0.01, ***p<0.005).*

DISCUSSION

Under physiological conditions IL-10 is often produced and secreted together with other immune mediators that can positively, negatively or synergistically impact pure IL-10-mediated effects. TLR9-triggered pathway potently induces IL-10 production in PBMC-derived purified human B cells cultures.[204] However, under this condition secreted IL-10 production is accompanied with production of a wide range of other molecules.

Our aim was to investigate direct effects of IL-10 expression in B cells. Normal human B cells have been toughly resistant to most of the gene transfer techniques. Among known transfection methods of non-viral vectors nucleofection was the most efficient to introduce plasmid DNA into human pre-B cells.[206] For the first time we established, optimized and demonstrated efficient expression plasmid vector mediated IL-10 gene transfer and consequent IL-10 overexpression using nucleofection in primary human peripheral B cells. This experimental model served us to investigate phenotype and immunoregulatory potential of IL-10 overexpressing B cells on different types of immune responses.

Transfection of the human IL-10 gene into B cells led to a rapid (overnight) and high expression of IL-10 and SOCS3, which is a direct IL-10-responsive gene,[207] and can serve as a control for overexpression of biologically active IL-10.[207] Production of proinflammatory cytokine TNF-α,[208] and chemokines IL-8 and MIP-1α,[189] strong chemoattractants for neutrophils and T_H1 cells, were significantly decreased while concentrations of anti-inflammatory IL-1Ra[209] and VEGF were significantly elevated in IL-10-overexpressing B cell supernatants when compared with control transfected B cells samples. Relative to control samples, IL-10-overexpressing B cells acquired an immunoregulatory phenotype as early as 24h post-transfection characterized by augmented GARP and IL2RA mRNA levels, both molecules known to be upregulated on regulatory T cells.[210 211] IL-10-overexpressing B cells partially share a pattern of surface molecules preferentially expressed on previously described human regulatory IL-10-producing Br1 cells.[204] To be distinguished from $CD24^{hi}CD38^{hi}$ cells[159] and relative to control transfected cells, IL-10-overexpressing B cells express more CD38 but not CD24. When compared with $CD25^{+}CD71^{+}CD73^{-}$ Br1 cells IL10-overexpressing B cells[204] express more CD25 (protein level), while CD71 and CD73 levels did not change upon high IL-10 when compared with their control counterparts. There was a trend for higher CD5 but not CD1d observed along with IL-10 overexpression, which partially resembles the phenotype of mouse regulatory $CD1d^{hi}CD5^{+}$ IL-10-producing cells.[139] These data suggest that solely IL-10 may have influence on direct or indirect induction of CD25, CD38 and CD5 in human IL-10-producing B cells while higher expression of CD24, CD71, CD73 may require either longer IL-10 exposure (possible delayed IL-10-indirect effect) or additional signals triggered beyond IL-10 (upon naturally IL-10-eliciting conditions, e.g. TLR-9).

Early changes (24h) in the expression of transcription factors included upregulation of IRF-4 and XBP-1, which are both characteristic for memory B cell transition to plasma cell before terminal differentiation, which requires BLIMP-1 upregulation.[212]

Furthermore, access to high IL-10 in B cells caused significantly decreased CD80, and a tendency for CD86 downregulation. At the same time high HLA-DR expression was retained as well as higher transcription rate for PD-L1 gene rendering these cells' profile to a more readily antigen-presenting one with lower costimulation capacity. IL-10-overexpressing B cells significantly downregulated expression of CD19, which is the feature of activated B cells but also decreased CD27 expression (both changes persisted 6 days after transfection regardless of additional TLR9-L stimulation (data not shown)). Our experimental set-up employed total peripheral blood CD19+ cells containing both naïve and memory B cells. 36h after transfection with IL-10 there were significantly more total IgD+ cells and less total IgM+ cells with a tendency for an increase in the naïve IgM+IgD+ population the effect which in combination with reduced CD27 and increased CD38 expression suggests two possible B cell fates: more naïve-like B cells[213] and "activated" ones underwent class- switch to IgA/IgG/IgE plasmablasts. The overall reduction in transcripts for AID and IgA after 72h suggests less class-switching to occur. Taken together, a condition with high levels of IL-10 expression supports possible "two lines" of B cell fate: one resembling immature transitional–like B cells and the other class-switched plasmablasts. Secreted immunoglobulins levels reflect a tendency for the IgE downregulation as a consequence of high IL-10 access. Since we did not observe elevated IgG4 production, additional signals alone or synergistic with IL-10 (e.g. TLR9 pathway-induced) seem to be required for an increase in secreted IgG4.[204]

PBMC co-cultured with IL-10-overexpressing B cells and stimulated with either TLR2-L or TLR4-L produced less proinflammatory cytokines (TNF-a, IL-1b and IL-6), as well as IL-8 and G-CSF than control transfected B cell co-cultures, indicating a substantial anti-inflammatory role of IL-10-producing cells possibly mediated by IL-10-mediated direct or indirect actions (SOCS3, IL1Ra). At least in part this effect may be explained by the impact of IL-10-overexpressing B cells on MDDC that extends from inhibition of cytokine release to suppression of their maturation downregulating their overall co-stimulatory potential (reduced CD80, CD86 and CD83, and induced PD-L1

surface expression) and therefore rendering MDDC toward regulatory phenotype.

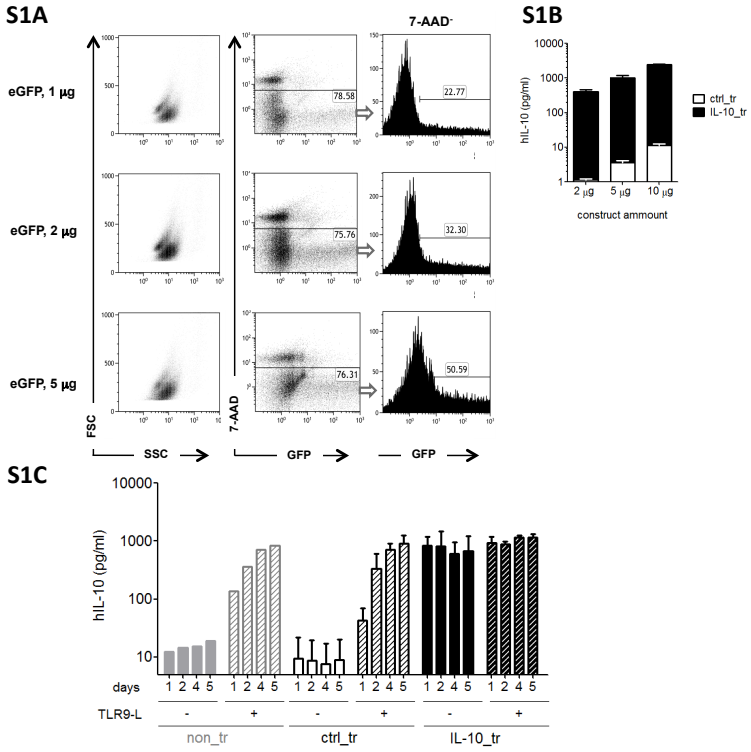
Although even the total fraction of non-manipulated B cells co-cultured with PBMC showed a trend for inhibition of their specific proliferation induced by TT, IL-10 overexpressing B cells showed the capacity to reduce it down to a minimum, which goes in line with previously described findings.[204, 214]

Taken together, when exposed to high IL-10 due to overexpression B cells quickly acquire complex immunoregulatory-like phenotype characterized by increase in SOCS3, GARP and IL2RA expression all three molecules described to be highly expressed in regulatory T cells long with persistent downregulation of CD19 and CD27 and increased CD1d, CD5 and CD38 expression, decreased capacity of co-stimulation. In addition, IL-10-overexpressing B cells are able to exert remarkable suppressive effects on both arms of the immune system: acting directly on mechanisms of rapid acting innate immunity (inhibiting release of cytokines' elicited via TLRs in primarily antigen presenting cell portion of PBMC) and on professional antigen-presenting cells, reducing their maturation profile through reduction of cytokine production and co-stimulatory potential, therefore affecting initiation and fate of adaptive immune response. IL-10 producing B cells are particularly potent in inhibiting antigen-specific proliferative response *in vitro* supporting the role of antigen specific IL-10 producing B cells limiting the memory T responses *in vivo*.

These data suggest that solely IL-10 provision to B cells is sufficient for a change in its profile toward a complex immunoregulatory phenotype, which, in conjunction with secreted IL-10 in co-cultures with autologous immune cells, is capable to exert substantial anti-inflammatory functions. These findings confirm the importance of IL-10-producing B cells regulating different aspects of immune response, particularly their effect on dendritic cells and bring better understanding of the capacity of solely IL-10 on B cell physiology and

regulatory profile applicable for their specific targeting in the fields of allergy and asthma, autoimmunity and tumor immunity.

Supplementary figures



Supplementary Figure 1. Optimization of B cell transfection efficiency and kinetics of IL-10 expression. *B cells were transfected with indicated ammounts of either pmaxGFP construct or pORF-hIL-10 (IL10_trB) or pORF-mcs (ctrl_trB) plasmid and cultured in medium for 24h. Transfection efficiency was determined by flow cytometry and presented as (**A**) proportion of live GFP+ cells and as (**B**) secreted IL-10 determined by specific ELISA in cell culture supernatants of transfected cells. (**C**) After transfection B cells were either cultured in medium only or stimulated with 1 µM TLR9-L during the period of 5 days. Secreted IL-10 was measured by specific ELISA in cell culture supernatants (n=2)*

6.3 Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligonucleotides.

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Short Title: TLR- induced B cell activation is suppressed by telomeric ODN

Original article

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Abstract

Background: The fine balance of immunoglobulins (Ig) E, IgG1, IgG4 and IgA in healthy production is maintained by the interaction of B cells with adaptive and innate immune responses. The regulation of toll-like receptors (TLR)-driven innate and adaptive immune effector B cell responses and the role of mammalian telomeric TTAGGG repeat elements represent an important research area.

Methods: Human PBMC and purified naive and memory B cells were stimulated with specific ligands for TLR2, 3, 4, 5, 7, 8 and 9 in the presence or absence of telomeric oligonucleotides. B cell proliferation, differentiation and antibody-production were determined.

Results: TLR9 ligand directly activates naive and memory B cells, whereas TLR7 can stimulate them in the presence of plasmacytoid dendritic cells. Human B cells proliferate and turn into antibody-secreting cells in response to TLR3, TLR7 and TLR9, but not to TLR2, TLR4, TLR5 and TLR8 ligands. Stimulation of B cells with intracellular TLR3, TLR7 and TLR9 induced an activation cascade leading to memory B cell generation and particularly IgG1, but also IgA, IgG4 and very low levels of IgE production. Mammalian telomeric ODN significantly inhibited all features of TLR ligand-induced events in B cells including B cell proliferation, IgE, IgG1, IgG4, IgA production, class switch recombination, plasma cell differentiation induced by TLR3, TLR7 and TLR9 ligands.

Conclusion: B cells require specific TLR stimulation, T cell and plasmacytoid dendritic cell help for distinct activation and Ig production profiles. Host-derived telomeric ODN suppress B cell activation and antibody production demonstrating a natural mechanism for the control of over exuberant B cell activation, antibody production and generation of memory.

Key words: B cell, IgA, IgG1, IgG4, IgE, plasmacytoid dendritic cell, telomeric ODN, toll-like receptor ligand

Introduction

The fine balance of immunoglobulins (Ig) E, IgG1, IgG4 and IgA is deviated to exuberant production of allergen-specific IgE and weak production of IgG4 in allergic individuals.[215] The interaction of environmental factors including allergens and microbial components with the innate and adaptive immune system results in class switch recombination of immunoglobulins.[216] Cells of the innate immunity equipped with Toll like receptors (TLR) have an immense capacity to recognize a diverse spectrum of microorganisms including bacteria, viruses, fungi and parasites.[217, 218] While a TLR10 ligand is yet to be described in humans, the specific ligands of TLR1 to TLR9 drive innate and adaptive immune effector cells towards inflammation and microbicidal activity.[217, 218] TLR1, 2, 4, 5 and 6 are expressed on the cell surface, whereas TLR3, 7, 8 and 9 are expressed in the endosomes of cells.[219-222] The intracellular localization of the latter TLRs allows bacteria and virus to be degraded in the endosomes/lysosomes after engulfment and phagocytosis in innate immune cells during infection. These processes are induced by the association of double stranded RNA to TLR3, single stranded RNA to TLR7 and TLR8 and unmethylated CpG motifs containing DNA to TLR9.[220, 223-228] TLR distribution intensity in the immune system cells and ligands for TLR has a critical role to understand host-pathogen interactions and the effects on the adaptive immunity. TLR expression differs between mouse and human cells, especially in B cells. Mouse B cells proliferate and differentiate upon LPS and CpG stimulation[229, 230], whereas human B cells respond to CpG[231] but not to LPS stimulation.[25, 232]

Bacterial DNA contains immunostimulatory CpG motifs that stimulate dendritic cells, NK cells and B cells and lead to their proliferation, maturation and secretion of a variety of cytokines, chemokines and immunoglobulins.[218, 233, 234] It has been reported that suppressive oligonucleotides (ODN) motifs can selectively block CpG-mediated immune activation. Suppressive motifs are rich in poly “G” or “GC” sequences and are present in the DNA of mammals and certain viruses. Suppressive ODN-expressing arrays of the hexameric TTAGGG repetitive elements patterned after mammalian telomeres[235, 236], has

been demonstrated to block CpG-induced cytokine production *in vitro* and inhibit CpG-induced inflammatory reactions such as lethal endotoxic shock in mice.[237, 238]

Therefore, we postulated that a natural inhibitory mechanism mediated by mammalian telomere motifs could help to restore B cell homeostasis. Stimulation of human PBMC via endosomal associated TLRs as well as direct activation of naive or memory B cells with CpG ODN and plasmacytoid dendritic cell maturation by TLR7 induces polyclonal B cell activation, proliferation and IgE, IgA IgG1 and IgG4 production suggesting a mechanism of immunological memory. After class switching in naive B cells, memory B cells and plasma cells that produce immunoglobulin E (IgE^+ cells) develop through a germinal-center IgE^+ intermediate cell without an IgG1 phase. In addition, cellular IgE memory resides in IgE^+ memory B cells, and $IgG1^+$ memory B cells are not an important source of IgE memory.[239] Here, we demonstrate that a synthetic analogue of mammalian telomeres containing four repeats of TTAGGG motif suppresses B cell activation, proliferation and antibody production induced directly or indirectly by TLR3, TLR7 and TLR9 ligands.

Materials and Methods

Isolation and purification of cells. PBMC were obtained from heparinized whole blood or buffy coats from healthy individuals by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation. Cells were washed three times and resuspended in RPMI 1640 medium supplemented as previously described.[240] B cells were purified by magnet-activated cell separation after labeling of cells with anti-CD19 microbeads (MACS, Miltenyi Biotec AG, Bergisch Gladbach, Germany). The purity of B cells was >94% as assessed by flow cytometric analysis of cells stained with ECD-labeled anti-CD19 mAb (Immunotech, Beckman Coulter, Marseille, France).

Following B cell staining with anti-CD19 mAb (Dako AG, Baar, Switzerland) and anti-CD27 mAb (Immunotech, Beckman Coulter), naive and memory B

cells were double sorted by flow cytometric sorter (FACS Vantage SE, Beckton Dickenson, Pharmingen, Basel, Switzerland). The purity of naive and memory B cells was >99% as assessed by flow cytometric analysis of cells stained with PE-labeled anti-CD19 mAb (Dako AG, Baar, Switzerland), PC5-labelled anti-CD27 mAb (Immunotech, Beckman Coulter) and FITC-labeled anti-CD123 mAb (Miltenyi Biotec AG). Naive B cells contained <0.1% $CD27^+$ memory B cells and <0.5% plasmacytoid dendritic cells and, memory B cells contained <1% $CD27^-$ B cells and <0.9% plasmacytoid dendritic cells.

Plasmacytoid dendritic cells were isolated from PBMC by positive selection by using antibodies against BDCA-4 coupled to microbeads (FcRγ)-blocking reagent and the autoMACS magnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cultures of PBMC and B cells. Ligands for TLRs were Pam3-Cys-Ser-$(Lys)_4$ for TLR2 (Calbiochem AG, Laufelfingen, Switzerland), poly(I:C) for TLR3 (Amersham Biosciences, Lausanne, Switzerland), E. Coli derived lipopolysaccharide for TLR4 (Sigma Chem. Co. Buchs, Switzerland), flagellin for TLR5 (Calbiochem AG), 3M-001 for TLR7 (3M Pharmaceuticals, Minneapolis, USA), 3M-002 for TLR8 (3M Pharmaceuticals) as described previously [241], CpG2006 for TLR9 (Microsynth GmbH, Balgach, Switzerland). Phosphorothioate-modified ODNs were synthesized at the Microsynth GmbH, Balgach, Switzerland. Sequences of the phosphorothioate ODNs used were as follows: CpG2006 ODN: 5'-TCGTCGTTTGTCGTTTTGTCGTT-3', control for immunostimulatory ODN (non-CpG): 5'-ATGCACTCTGCAGGCTTCTC-3', telomeric ODN: 5'-TTAGGGTTAGGGTTAGGGTTAGGG-3', base scrambled "control ODN": 5'-ACATAGTCTCTCTCTAGTCAGTCT-3'. All reagents and ODNs used in this study contained <0.1 U/mg endotoxin. There was no cell toxicity of the ODN as investigated by annexin-V and 7-amino actinomycin D staining after 48 h up to 10 µM doses [242]. Recombinant IL-2 and IL-4 were from Novartis, Basel, Switzerland. Soluble CD40 ligand (sCD40L) was obtained from the transfected cell-line 8-40-1 (sCD40L-CD8-α fusion protein; Institute for Immunology, Basel, Switzerland) [243].

5×10^5 PBMC or purified naive or memory B cells were stimulated in 500 µl with different TLR-ligands in 48-well plates (Costar Corporation, Cambridge, MA). After initial experiments for dose optimization Pam3-Cys-Ser-$(Lys)_4$ was used at 0.6 µM, poly(I:C) at 25 µg/ml, lipopolysaccharide at 100 ng/ml, flagellin at 1.8 µM, 3M-001 and 3M-002 at 1 µM, CpG2006 and control ODN at 1 µM. IL-2 was used at 4 ng/ml, IL-4 was 25 ng/ml, sCD40L was at 20% v/v as supernatant[65], IFN-α and IFN-β were 100 ng/ml where indicated. TTAGGG telomeric ODN and control ODN were used in titrated doses and 1 µM in other experiments. Cells were pulsed with 1 µCi/well [^{3}H] thymidine (Dupont and NEN Life Science Products, Basel, Switzerland) for the last 8 h of incubation, and [^{3}H] thymidine incorporation was performed at day 3 for transformed B cells and T cells and at day 5 for primary B cells.

Analysis of B cell activation and proliferation. Isolated PBMC from buffy coat were stained with 5 µM carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes Europe BV Leiden, The Netherlands) for 12 min at 4^0C and then washed twice with complete medium. For the analysis of lymphocyte subset proliferation, 5×10^4 cells were stained with ECD-conjugated anti-CD19 mAb, PE-conjugated anti-CD4, PE-conjugated anti-CD8 or PE-conjugated anti-CD16 (Immunotech, Beckman Coulter) for 30 min at room temperature. For the analysis of B cell differentiation, $5x10^4$ cells were stained with ECD-conjugated anti-CD19 mAb together with anti-CD20 or anti-CD23-PE (Serotec Immunological Excellence, Raleigh, NC) or anti-CD27-PE or anti-CD38-FITC (Immunotech, Beckman Coulter) or anti-CD80-FITC or anti-CD86-PE or anti-BAFF-R-FITC (Beckton Dickenson, Pharmingen) or anti-TACI-PE (R&D Systems, Abington, UK) or anti-BCMA-FITC (Alexis Corp. Lausen, Switzerland) for 30 min at 4^0C or room temperature and washed with PBS. Stained cells were fixed in 2% paraformaldehyde (Sigma). The isotype controls were FITC, PE or ECD-conjugated mouse IgG1/IgG2 (Immunotech, Beckman Coulter). Plasmacytoid dendritic cells were co-cultured with allogeneic naive B cells or memory B cells at a ratio of 1:10 with IL-2 for 5 days in 200 µL in 96 well plates.

Determination of IgG1, IgG4, IgA and IgE and Ig-secreting cells. Total IgG1, IgG4, IgA and IgE were measured in culture supernatants at day 12 by ELISA as described.[63] Anti-human IgG1, clone JDC-1 (Beckton Dickenson, Pharmingen) and biotinylated anti-IgG1 (Beckton Dickenson, Pharmingen) were used to quantify IgG1. The detection limit of the IgG1 assay was 16 ng/ml human IgG standard (Calbiochem AG, Luzern, Switzerland). For total IgA determination anti-IgA1/2 mAb (Beckton Dickenson, Pharmingen) was used for coating and biotinylated anti-IgA1/2 mAb (Beckton Dickenson, Pharmingen) was used for detection. The detection limit of the IgA assay was 16 ng/ml and human IgA standard was from Sigma Chem Co.

Ig-secreting cells were detected by isotype-specific enzyme linked immunospot assay (ELISPOT) on day 11 of culture. 96-well ELISPOT plates (Pall Corporation, MI, USA) were coated with the same mAb used for ELISA. After washing with PBS 0.1 % tween and blocking with PBS with 2% skimmed dry milk (Euroclone, Milano, Italy). 3 x 10^4 pre-stimulated PBMC from each condition were incubated in 200 µl of medium 96-well flat bottom ELISPOT plates in duplicate for 24 hours at 37^0C. Locally produced IgG1, IgG4, IgA and IgE were captured by anti-IgG1 (Beckton Dickenson, Pharmingen), anti-IgG4 (Skybiolimited, Bedfordshire, UK), anti-IgA1/2 mAb (Beckton Dickenson, Pharmingen), anti-IgE. The immunoglobulin specific mAb were detected by a secondary biotinylated/detection antibody for IgG1, IgA and IgE (Beckton Dickenson, Pharmingen) followed by washings and incubation with streptavidin conjugated to alkaline phosphatase. For the development of IgG4 spots alkaline phosphatase-conjugated anti-human IgG was used (Sigma Chem Co.) Colored purple spots developed after substrate addition (nitroblue tetrazolium and bromo-chloro-indolyl phosphate in dimethyl formamide, Bio-Rad Lab. Inc. CA, USA), and were counted by an automated software (ImmunoSpot; C.T.L. Europe GmbH. Aaelen, Germany). Spot size bigger or smaller than average +/- 3 standard deviation was ignored. After 10 days of cell culture with TLR ligands, 24 hours of incubation of the cells in ELISPOT

plates for the secretion of immunoglobulins was found to be the optimal time for the determination of frequency of immunoglobulin-producing cells.

Immunoglobulin mRNA transcription determination. Total RNA was extracted from the PBMCs using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Approximately 500 ng total RNA was reverse transcribed by ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, USA) into complimentary DNA (cDNA). The PCR primers and probes were designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Applied Biosystems, Foster City, CA, USA). A reference gene screening has been carried out, and elongation factor (EF)-1α was used as an endogenous control. Primers used for relative quantification AICDA, and Iγ-Cγ1, V_HDJ_H-Cγ4, Iε-Cε, Iα1/2-Cα1/2 RNA transcripts are shown in the Table 1S.

The prepared cDNAs were amplified by using iTaq SYBR Green Supermix with ROX (Bio-Rad, Basel, Switzerland), according to the manufacturer's recommendations in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) in triplicates. Relative quantification and calculation of the range of confidence was performed by using the comparative $\Delta\Delta$CT method.[244]

***Table 1S**. Forward and reverse primer sequences (5'-3') for AICDA, and Iγ-Cγ1, V_HDJ_H-Cγ4, Iε-Cε, Iα1/2-Cα1/2 RNA transcripts.*

mRNA	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
AICDA	AGAGGCGTGACAGTGCTACA	TGTAGCGGAGGAAGAGCAAT
Iγ-Cγ1	CTCTCAGCCAGGACCAAGGA	GGTGGGCATGTGTGAGTTTTG
V_HDJ_H-Cγ4	ACCATGGTCACCGTCTCCTCA	GGGACCATATTTGGACTC
Iε-Cε	ACACATCCACAGGCACCA AA	TTGCAGCAGCGGGTCAA
Iα1/2-Cα1/2	CGCTGGCCTTCACACAGA A	CGCCATGACAACAGACACA

Statistical Analysis. Results are shown as mean ± SD. Nonparametric statistical comparison between groups was performed by Mann Whitney U test. The number of donors indicated in the figure legends includes all donors analyzed in the relevant experiment.

Results

TLR3, TLR7 and TLR9 stimulations induce B cell proliferation, which is suppressed by telomeric ODN

Activation and clonal expansion of B cells is an essential event for immunoglobulin production. To investigate the role of different TLRs on human B cell activation, proliferation and different isotypes of immunoglobulin production, we used different TLR-stimulated PBMC, purified B cells and naive and memory B cell subsets isolated from peripheral blood of healthy humans. First, PBMC were stimulated with ligands of TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 in the presence of IL-2 for five days. [^{3}H] thymidine incorporation was determined after 5 days. Ligands for TLR3, TLR7 and TLR9 induced significant cell proliferation. Human telomeric ODN significantly suppressed TLR3-, TLR7- and TLR9-mediated PBMC proliferation in a dose dependent manner (Fig. 1A) reaching up to 60 %, 90% and 80% inhibition at 5 μM doses, respectively (Supporting information Fig. S1A). For comparison, control ODN did not show significant suppressive activity (Fig. S1B). Telomeric ODN, 1 μM, significantly suppressed PBMC proliferation stimulated by TLR3, TLR7 and TLR9 ligands (Fig. 1B).

The next step was to identify the proliferating cell type within PBMC upon TLR3, 7 and 9 stimulations. Interestingly, stimulation of TLR3, 7 and 9 induced $CD19^+$ B cell, but not $CD4^+$, $CD8^+$ T cell or $CD16^+$ NK cell proliferation (Fig. 2). Mammalian telomeric ODN significantly inhibited B cell proliferation induced by TLR3, 7 and 9.

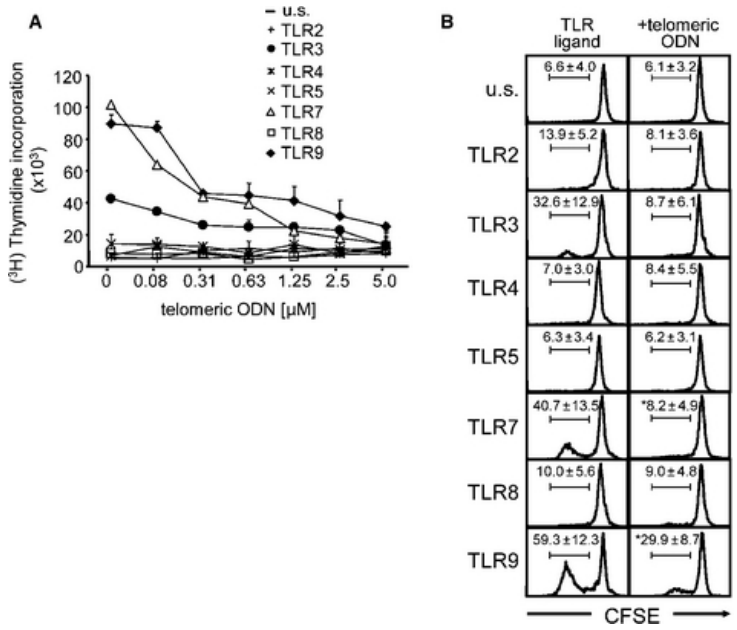

FIGURE 1. *Suppression of TLR-induced human peripheral blood mononuclear cell (PBMC) proliferation.* ***A****, Suppression of TLR-induced PBMC proliferation at different concentration of telomeric oligodeoxynucleotide (ODN). Human PBMC 10^6/ml were stimulated with ligands for TLR2 (0.6 µM, Pam3-Cys-Ser-(Lys)4), TLR3 (25 µg/ml, poly I:C), TLR4 (100 ng/ml, lypopolysaccharide), TLR5 (1.8 µM, Flagellin), TLR7 (1 µM, 3M-001), TLR8 (1 µM, 3M-002) and TLR9 (1 µM, CpG2006) in the presence of 4 ng/ml IL-2. Telomeric ODN was titrated 1:2 starting at 5 µM. To determine proliferative responses on day 5, cells were pulsed with 1µCi/well [3H] Thymidine and [3H] Thymidine incorporation was determined after 8 hours.* ***B****, To further analyze the effects of TLRs and telomeric ODN on PBMC proliferation the decrease in CFSE intensity of the cells was measured by flow cytometry and was used as a measure of the extent of cell division. Human PBMC from healthy donors were labeled with CFSE and stimulated with TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 ligands in the presence of IL-2 with and without 1 µM of telomeric ODN. TLR-induced cell proliferation stained by CFSE was determined on day 5 with flow cytometry. Compared to the unstimulated control group, TLR3, TLR7 and TLR9 ligands induced significant cell proliferation. Even at higher doses used in preliminary experiments TLR2, TLR4 TLR5 and TLR8 ligands did not induce detectable cell proliferation in human PBMC. Mean ± standard deviation of percentage of proliferating cells in 5 different experiments is shown in each histogram. Telomeric ODN significantly suppressed cell proliferation in all conditions (P <0.01).*

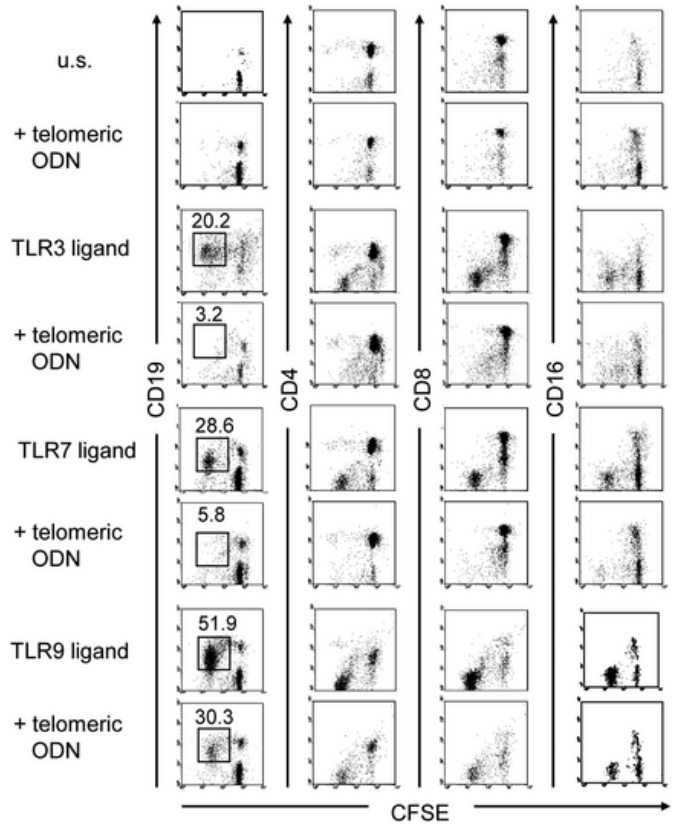

Figure 2. Telomeric ODN inhibits B-cell proliferation induced by TLR3, TLR7 and TLR9. Human PBMCs from healthy donors were labeled with CFSE and stimulated with ligands for TLR3, TLR7 and TLR9 in the presence of IL-2 with and without telomeric ODN at the concentrations indicated in the Fig. 1. Cells were counterstained with CD19, CD4, CD8 and CD16. The numbers in the boxes represent the percentage of the proliferating CD19-stained cells. One representative of four experiments is shown. Telomeric ODN significantly suppressed $CD19^+$ cell proliferation in all conditions ($P < 0.01$*). In these experiments, IL-2 consistently induced the expansion of B cells with these three stimuli in PBMCs. IL-2 also induced the proliferation of a small fraction of CD4, CD8 and CD16 positive cells. This fraction was more visible in $CD4^+$ cells, however, was always less than 4.5% of the cells and was not suppressed by telomeric ODN.*

The exact identification of B cell subsets is instrumental to understand their dynamics under physiological and pathological conditions. Human memory B cells are currently identified according to the expression of CD27, which is absent on naive B cells. It was shown that naive and memory B cells respond differently to the same TLR ligand especially to CpG[25] In addition, it was recently demonstrated that under certain conditions including cell-cell contact and appropriate cytokine and growth factor support, $CD27^-$ naïve B cells increase TLR9 expression, proliferate in response to CpG stimulation without

B cell receptor signaling and differentiate into plasma cells.[245] To specifically differentiate the proliferative responses of naive and memory B cells induced by TLR3, 7 and 9 stimulation, highly purified $CD19^+CD27^-$ naive and $CD19^+CD27^+$ memory B cells were stimulated. Upon TLR9 stimulation naïve and memory B cells proliferated without the help of type 1 IFNs or plasmacytoid dendritic cells (Fig. 3A, B). TLR3 and TLR7 ligands, however, failed to directly induce the proliferation of highly pure naive and memory B cells. TLR7 stimulated a small fraction of naïve B cells in the presence of plasmacytoid dendritic cells, and memory B cells in the presence of IFN-α, IFN-β or plasmacytoid dendritic cells. Again, mammalian telomeric ODN significantly suppressed TLR7- and TLR9-ligand-induced $CD19^+CD27^-$ naive and $CD19^+CD27^+$ memory B cell proliferation independent of the combination with IFN-α, IFN-β and the presence of plasmacytoid dendritic cell help.

These data demonstrate direct and indirect activation and clonal expansion of B cells by TLR stimuli. TLR3 and TLR7 ligands cannot directly stimulate naive and memory B cells. In a mixed population of PBMC, when they coexist with the T cell help or dendritic cells, they become capable of B cell stimulation. As a strong stimulator for B cells, CpG induces direct activation of memory and a fraction of naive B cells. TLR7 stimulates B cells in the presence of IFN-α, IFN-β secreted by the plasmacytoid dendritic cells.

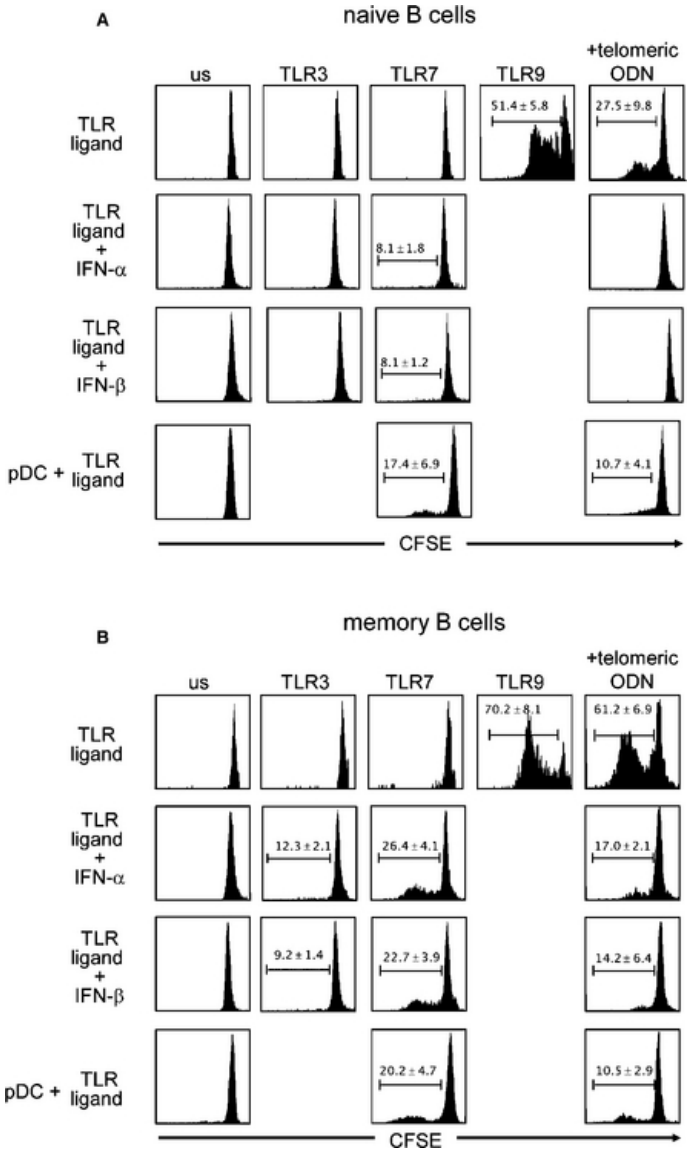

Figure 3. Distinct stimulation of naive and memory B-cell proliferation by TLR3, TLR7 and TLR9 ligands and suppression by telomeric ODN. Purified $CD19^{+}CD27^{-}$ naive and $CD19^{+}CD27^{+}$ memory B cells were labeled with CFSE to investigate their proliferation by TLR3, TLR7 and TLR9 stimulation with and without interferon-alpha (100 ng/ml), interferon-beta (100 ng/ml). Human plasmacytoid dendritic cells 4×10^{4} were added to naïve and memory cells. (A) TLR9 ligand induce the proliferation of naive B cells. The addition of plasmacytoid dendritic cells induces the proliferation of naïve B cells by TLR7 ligand. (B) High proliferation rate of memory B cells with CpG (TLR9 ligand) and moderate proliferation by TLR7 ligand with IFN-α, IFN-β and the addition of plasmacytoid dendritic cells. The proliferation rates of naïve and memory B cells were suppressed by telomeric ODN. One representative of three experiments is shown.

Regulation of class switch recombination and IgE, IgG1, IgG4, IgA production by TLR3, TLR7, TLR9 ligands and telomeric ODN

B cells are continuously exposed to substances that stimulate the innate immune response from commensal flora bacteria in respiratory and gastrointestinal mucosae and they produce immunoglobulins against pathogens. After demonstration of the difference in TLR3-, 7- and 9-ligand-induced direct or indirect B cell proliferation, we examined the effects of these ligands on AICDA mRNA expression and immunoglobulin synthesis by B cells and PBMC as well as the role of IL-2, IL-4 and sCD40L stimulation.

PBMC were stimulated with ligands for TLR3, 7 and 9 in the presence and absence of telomeric ODN and control ODNs. IL-2, IL-4 and sCD40L were used to mimic T cell help and promote B cell clonal expansion. IgE, IgG1, IgG4 and IgA were measured on day 5 cell lysates (as mRNA) and day 12 culture supernatants (Fig. 4A, B, C). IgE antibody synthesis and Iε-Cε RNA transcripts expression was induced by TLRs in combination with IL-4 and sCD40L, but not induced with IL-2 and sCD40L in healthy individuals (data not shown).

As observed in B cell proliferation, the profiles of immunoglobulin production showed differences in PBMC and purified B cells (Fig. 4A, B and Fig. S2). In PBMC, stimulation via TLR3, 7 and 9 even in the absence of IL-2 induced IgG1, IgG4 and IgA, in detectable quantities. IL-2 significantly increased the synthesis of IgG1, IgG4 and IgA stimulated with TLR3, 7 and 9 ligands. Stimulation of cells with sCD40L had no significant effect on the production of immunoglobulins in the absence of IL-2, whereas IL-2 and sCD40L combination boosted IgG1 levels, and they had no additional contribution to the induction of IgG4 and IgA. Increased expression of Iγ-Cγ1, V_HDJ_H-Cγ4, Iα1/2-Cα1/2 RNA transcripts for IgG1, IgG4 and IgA in IL-2 and sCD40L-stimulated PBMC with TLR3, TLR7 and TLR9 verified our results (Fig. 4C). Consistent with the proliferation studies, telomeric ODN significantly suppressed IgE, IgG1, IgG4 and IgA secretion and their mRNA expression independent of the type of TLR ligand in the presence of IL-4, IL-2 and

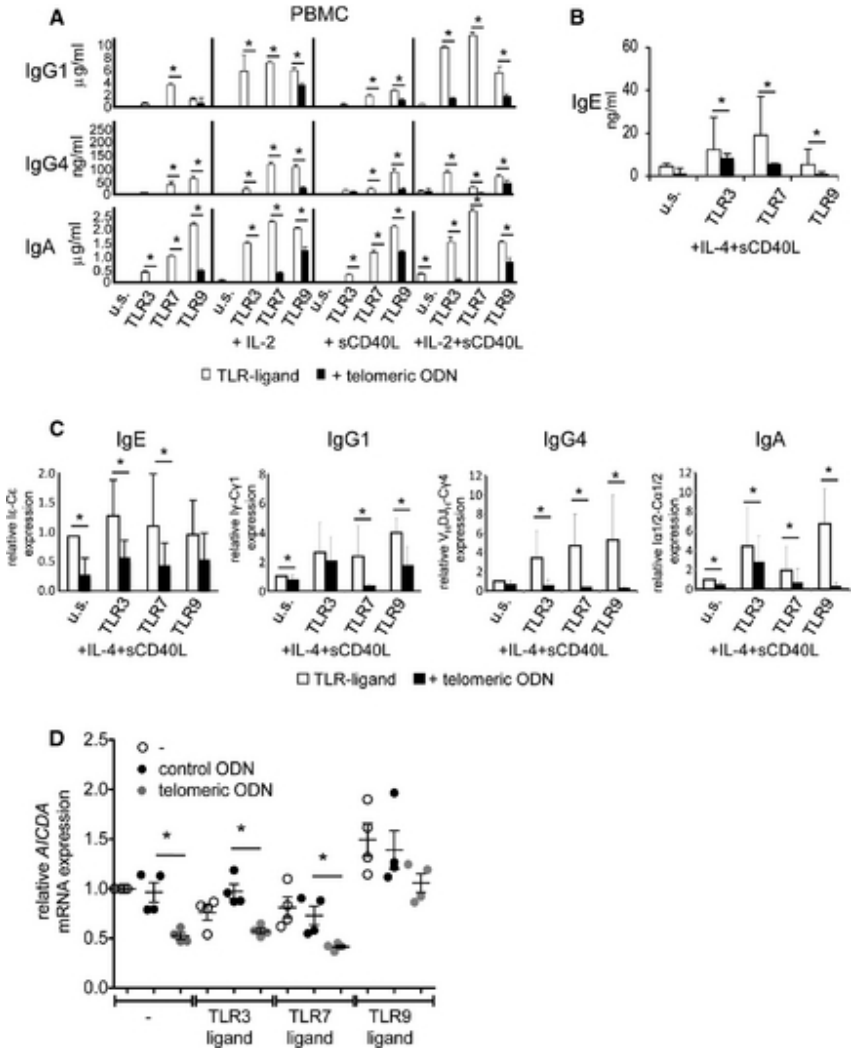

*Figure 4. Different requirements in IgE, IgG1, IgG4 and IgA production by PBMC with TLR3, TLR7 and TLR9 stimulation and their suppression by telomeric ODN. (A) IgG1, IgG4 and IgA immunoglobulin synthesis by PBMC 0.5 × 10^6/ml in 500 µl in 48-well plates was stimulated with TLR3, TLR7 and TLR9 ligands in the absence or presence of IL-2 and sCD40L or both. TLR7 and TLR9 ligands and telomeric ODN were used at 1 µM, and TLR3 ligand was used at 25 ug/ml. IgG1, IgG4 and IgA were measured in day 12 supernatants. (B) PBMCs 0.5 × 10^6/ml in 500 µl in 48-well plates were stimulated with TLR3, TLR7 and TLR9 ligands in the presence of IL-4 and sCD40L. IgE was measured in day 12 supernatants by ELISA. (C) Relative Iε-Cε, Iγ-Cγ1, V_HDJ_H-Cγ4, Iα1/2-Cα1/2 RNA transcripts for IgE, IgG1, IgG4 and IgA quantified with real-time PCR. The measurements after TLR ligand stimulations were expressed as fold increase relative to the measurement from unstimulated cells. Results are representative of four experiments. (D) Suppression of AICDA mRNA expression by telomeric ODN. PBMCs cultured with sCD40L + IL-2 were stimulated TLR3, TLR7 or TLR9 ligands alone or in combination with telomeric or control ODN. mRNA expression of AICDA was measured after 5 days and its expression is calculated relative to sCD40L+IL-2. Horizontal bar shows mean (±SEM). Results are representative of four independent experiments. *P < 0.05.*

sCD40L experimental conditions. Upon exposure to the infections or immunizations, activation-induced cytidine deaminase (AICDA) regulates class switch recombination and somatic mutation in B cells.[246] The expression of AICDA mRNA, was measured after 5 days (Fig. 4D). Telomeric ODN suppressed the expression of AICDA mRNA even in PBMC cultured with IL-2+sCD40L and in combination with TLR3, TLR7 and TLR9 ligands. Control ODN did not show any stimulatory or inhibitory effect on the effect of TLR ligand and telomeric ODN.

We then investigated the effect of TLR3, TLR7 and TLR9 stimulation on antibody forming cell frequency and whether it is also inhibited by telomeric ODN. Human PBMC were stimulated with TLR3, TLR7 and TLR9 ligands in the presence of telomeric ODN, IL-2 and sCD40L. Secreted immunoglobulins were captured on ELISPOT plates between day 10 and day 11 of cultures. Stimulation via TLR3, TLR7 and TLR9 significantly increased IgG1, IgG4 and IgA producing cell frequency (Fig. 5A). Particularly, a relatively high frequency of cells produced IgA after TLR3, TLR7 and TLR9 ligand stimulation. It has to be noted here that secreted immunoglobulins determined in the supernatants by ELISA represent a cumulative value after 12 days. However, ELISPOTS represent the number of Igs secreting cells in a certain time fragment within the course of cultures. Telomeric ODN significantly suppressed the number of antibody-forming cells producing IgG1, IgG4 and IgA in all experiments (Fig. 5B) supporting the data obtained by ELISA.

Suppression of TLR3, TLR7 and TLR9 ligand induced B cell differentiation by telomeric ODN

Since B cells undergo differentiation towards plasma cells to produce immunoglobulins, we next aimed to investigate, whether B cell differentiation is affected by TLR ligands and whether telomeric ODN influence B cell differentiation. Human PBMC were double stained with mAbs for CD19, CD20, CD27, CD38, CD80, CD86, B cell activating factor of the TNF family (BAFF)-receptor, transmembrane activator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA), which are expressed on B cells

depending on the stage of maturation and activation of the B cell. Stimulation of PBMC via TLR3, TLR7 and TLR9 in the presence of IL-2 resulted in a significant increase in the numbers of $CD19^+$ B cells. These proliferating B cells expressed high levels of CD27, CD38, CD86 and, demonstrating that a fraction of proliferating B cells stayed as memory B cells and their APC function was increased and some of them matured to antibody-forming cells (Fig. S3A). The formation of memory B cells ($CD19^+CD27^+$) was significantly inhibited by telomeric ODN. This was in parallel to suppression of $CD20^+$, $CD86^+$ and CD38 $high^+$ B cells. The expression rate of BAFF-R, TACI and BCMA showed different patterns depending on TLR ligand types (Fig. S3B). TLR3 stimulation induced moderate increase in BAFF-R, TACI and BCMA expression, TLR7 stimulation induced TACI and BCMA expression and finally the expression rate of these B cell surface markers were not stimulated by

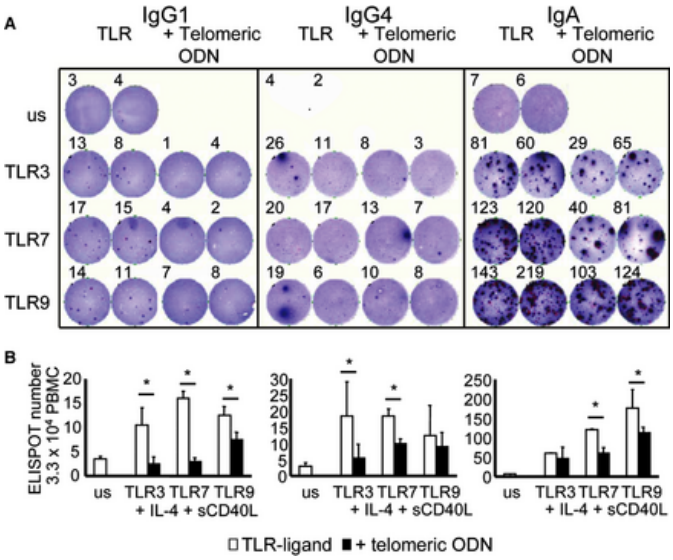

Figure 5. The number of TLR3-, TLR7- and TLR9-ligand-induced IgG1, IgG4 and IgA producing cells decreases by telomeric ODN. (A) Human PBMCs were stimulated with TLR3, TLR7 and TLR9 ligands with and without telomeric ODN. 3×10^4 PBMCs from each condition on day 10 were incubated for 24 h. The ELISPOTs of duplicate wells for each treatment condition on ELISPOT plates are shown. The numbers of spots in each well is indicated on upper left corner. (B) Results representative of four independent experiments are shown in the bar graphs. The open *bar represent TLR stimulation and the* filled *bars represent the addition of telomeric ODN for IgG1, IgG4 and IgA spots number. *P < 0.05.*

TLR9-ligand. $CD27^+$ and $CD86^+$ B cells development, $TACI^+$ and $BCMA^+$ B cells development were suppressed by telomeric ODN. In contrast, BAFF-R-expressing B cells were not suppressed, whereas BAFF-R-negative B cells showed a significant decrease by telomeric ODN.

Finally in order to evaluate the plasma cell differentiation in B cells, we determined surface CD138 expression in parallel to $CD19^+$ expression. Surface CD138 expression, which is a highly and specific marker of plasma cells, was determined in 10 days of PBMC cultures with sCD40L+IL-4 and stimulated by TLR3, 7 or 9 alone or in combination with telomeric or control ODN. Stimulation with TLR3 or TLR7 ligands did not induce detectable CD138 expression, but TLR9 ligand induced its expression at the level of 1.6% of $CD138^+CD19^+CD3^-CD16^-CD14^-7\text{-}AAD^-$ cells (Fig. 6A). Control ODN did not change the ratio of $CD138^+CD19^+$ cells induced by TLR9 ligand. However, telomeric ODN significantly suppressed the $CD138^+CD19^+$ plasma cell differentiation from B cells induced by TLR9 ligand (Fig 6B, C).

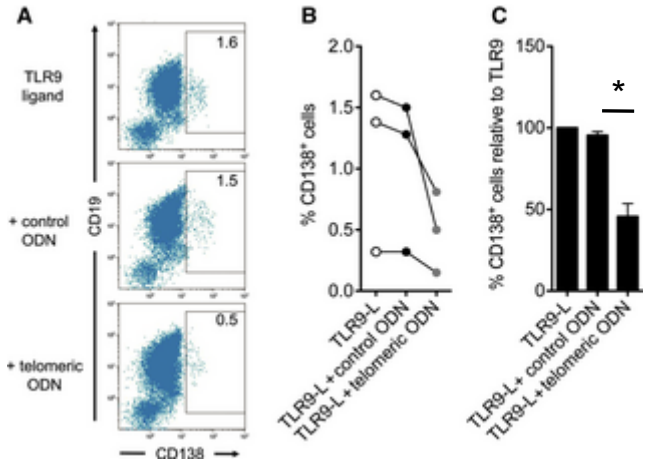

*Figure 6. Suppression of plasma cell differentiation by telomeric ODN. PBMCs cultured with sCD40L+IL-4 were stimulated TLR3, TLR7 or TLR9 alone or in combination with telomeric or control ODN. (A) Surface CD138 expression was measured by flow cytometry after 10 days culture. $CD3^-CD16^-CD14^-7\text{-}AAD^-$ cells were gated. Stimulation with TLR3 or TLR7 ligands did not induce detectable CD138 expression. One representative example is shown as dot plots. (B) Connected dots show percentage of $CD138^+$ from three independent experiments. (C) Bar graphs show mean (±SEM) percentages of $CD138^+$ cells relative to TLR9 ligand stimulated cells (set to 100%). Results are representative of three independent experiments, *$P < 0.05$.*

Discussion

The present study demonstrates that TLR3, TLR7, TLR9 have distinct stimulatory pathways for B cell activation and immunoglobulin production. We demonstrate here a synthetic analogue of mammalian telomeres suppresses direct and indirect effects of TLR3, 7 and 9 ligands as strong inducers of B cell proliferation, differentiation and immunoglobulin production.

Here, we report that in addition to direct stimulation of B cells via TLR9, stimulation of PBMC via TLR3 and 7 indirectly leads to human B cell proliferation and immunoglobulin production. Although TLR3, 7 and 9 are expressed at the same cellular location, in the endosomes, their expression rates differ depending on the type of the cells. In addition to TLR9, human peripheral blood B cells express high levels of TLR1, TLR6 and TLR10, intermediate levels of TLR7, and low levels of TLR2, and TLR4, but not TLR3 [247]. In the present study, we demonstrate that stimulation via TLR9 directly activates $CD27^+$ memory B cells and a fraction of $CD27^-$ naive B cells. As shown by Huggins et al.[245] CpG stimulates not only $CD27^+$ memory B cells, but $CD27^-$ naïve B cells under special *in vitro* conditions. The present study demonstrates that CpG stimulates highly purified naïve and memory B cells and this stimulation is potentialized in the presence of IL-2. Both TLR3 and TLR7 stimuli significantly induced B cell proliferation only when PBMC were stimulated, suggesting that there is an indirect effect on B cells mediated by other cells or mediators that are present within the PBMC population. The role of other factors was demonstrated in the present study by co-cultures of highly purified cells. The role of IL-2 is demonstrated as one of the essential factors for B cell proliferation and antibody production by TLR3 and TLR7 stimuli. It was recently shown that IFN-α released by plasmacytoid dendritic cells upon stimulation with TLR7 was responsible for B cell polyclonal expansion and differentiation into Ig-producing plasma cells.[248] In the present study, the proliferation of purified human B cells, particularly memory B cells in the presence of IFN-α, IFN-β and plasmacytoid dendritic cells induced by TLR7 shows that this stimulatory effect was mediated via plasmacytoid dendritic cells. The activation of plasmacytoid dendritic cells by TLR7 results

in the secretion of IFN-α and IFN-β that induce B cell activation, proliferation and IgE, IgG1, IgG4 and IgA production.

Whereas previous studies reported that TLR9 stimulation by CpG inhibited CD40/IL-4-driven IgE synthesis in human B cells and IgG1 and IgE synthesis in murine B cells.[249, 250] A recent study by Ozcan et al.[251] demonstrated that murine naïve B cells when stimulated by TLR9 ligand with the cooperation of CD40, TACI and IL-4 produce IgG1 and IgE. In human PBMC, stimulation via TLR3, 7 and 9 even in the absence of IL-2, IL-4 and sCD40L induced IgG1, IgG4 and IgA, in detectable quantities and IgE with the combination of IL-4 and sCD40L. In contrast to PBMC, purified human $CD19^+$ B cells produce IgG1, IgG4 and IgA upon TLR9 stimulation but not TLR3 and TLR7 (Fig. S2). The production of IgG1, IgG4 and IgA was increased by TLR3 and TLR7 in the presence of IL-2 and this response was boosted when combined with sCD40L, but sCD40L alone did not induce additive effect on purified B cells. The production of IgE by purified B cells was not induced by TLR3, TLR7 and TLR9 in the presence of IL-4 and sCD40L contrary to the findings in PBMC. The production amount of IgE by intracellular TLRs is not robust when compared to IgG1, IgG4 and IgA in PBMC and moreover was not detectable in human purified B cell cultures. This finding suggests that intracellular TLR ligands may have a critical role in changing the balance against IgE and more in the direction of IgG1, IgG4 and particularly IgA in allergic diseases.[65]

In the present study, we investigated step by step all aspects of B cell activation, proliferation, Ig class switch recombination, AICDA expression and plasma cell differentiation and demonstrated that mammalian telomeric ODN significantly suppressed all of the features. Suppression of B cell activation and Ig production by suppressor ODN represents an important way for the treatment of diseases related to B cell over activation. It can also be used for allergic diseases. Rituximab, a chimeric mAB to CD20 that efficiently eliminates circulating and overactive B cells that have CD20 on their surfaces and is therefore used to treat diseases characterized by high number of active B cells. A pilot study was already conducted in extrinsic atopic dermatitis

characterized by highly elevated concentrations of IgE and the early results of treatment with rituximab suggest that it may be a promising treatment option for patients with severe atopic dermatitis.[171, 252]

Previous studies have demonstrated that suppressor ODN possessing four repeats of TTAGGG motifs inhibits CpG-induced inflammation in mice.[170, 235, 253] Human telomeres consist of tandem repetitive arrays of the hexameric sequence TTAGGG.[236] The enzyme telomerase is a cellular ribonucleoprotein responsible for adding telomeric repeats onto the 3' ends of chromosomes and thus compensates for cell division-associated telomere loss.[254] Elongation of telomeres with the telomerase results in an extended lifespan of a cell. Therefore, it was suggested that the length of the telomere rather than telomerase itself is responsible for limiting cell proliferation. Suppressive activity of TTAGGG motifs has been demonstrated to correlate with the ability of the sequence to form G-tetrads.[235] Individual mammalian cells contain phosphodiester TTAGGG motifs and some of them including some synthetic analogues are in single-stranded form. Therefore, it can be expected that high cellular concentrations of TTAGGG elements with suppressive capacity can be released to microenvironment following host cell death or following cell division. It is possible that self-DNA released by injured cells could down-regulate TLR-mediated inflammation response.

Another way to control TLR responses is the TLR tolerance phenomenon, which is explained by the unresponsiveness of the immune cells to the same or different TLR ligands upon the occurrence of repeated or chronic stimulation through TLRs.[255] The tolerant state in macrophages and B cells was associated with reduced NF-κB, MAPK activation and c-Jun phosphorylation.[256] However, B cells may receive general signals through TLRs and specific signals through BCR. The prestimulation of B cells with TLR7 may result in reduced B cell proliferation and IgM secretion upon subsequent TLR7 restimulation, but simultaneous BCR signaling may prevent or reverse TLR7- or TLR9-mediated TLR tolerance in human B cells. This distinct response of TLRs and BCR to the TLR7 and TLR9 ligands may be a

potential way to study the effects of telomeric ODNs on B cell responses induced by TLR ligands. The TLR9-ligand is a nucleic acid sequence that may bind to TLR9 localized in the endosomes. Telomeric ODNs, which are also nucleic acid sequences may have a potential to regulate B cell response similar to the tolerance phenomenon that takes place by repeated or chronic stimulation through TLRs.

In both PBMC and B cell cultures, mammalian telomeric ODN suppressed TLR3, TLR7 and TLR9 ligand induced B cell proliferation without regarding direct or indirect stimulation. Whereas the generation of both TACI and BCMA positive and negative B cells were suppressed, the BAFF-R expressing B cells were resistant to suppression by telomeric ODN. Moreover telomeric ODN significantly suppressed the proliferation of the antibody forming cells. These findings were fully supported by demonstration of significant decrease in secreted Igs to the supernatants of PBMC and purified B cells by telomeric ODN. It remains to be elucidated whether the different levels of B cell memory and serum antibodies to different pathogens depends on the amount of cell death and released telomeric ODN to the microenvironment during exposure and generation of the immune response.

It has been suggested that environmental stimuli such as CpG maintain continuous polyclonal memory B cell activation, proliferation and differentiation.[257] In this way, a constant level of plasma cells and serum antibodies could theoretically be maintained throughout a human life span. Our study showed that in addition to CpG, TLR3 and TLR7 ligands have a capacity to induce B cell activation in PBMC, which was significantly enhanced by IL-2. These findings demonstrate that direct or indirect stimulation of human B cells through endosomal associated TLRs plays a pivotal role in polyclonal B cell activation and immunoglobulin production to maintain long-term serological memory in humans. Suppression of this response by host-derived telomeric ODN suggests a natural mechanism to overcome hyper activation of B cells and hyper production of immunoglobulins under chronic inflammatory conditions. The distinct effects of intracellular

TLR3, 7 and 9 and telomeric ODN on B cell activation, proliferation, AICDA expression, Ig class switch recombination, IgE, IgG1, IgG4, IgA production and plasma cell differentiation suggest a crucial role for the immunomodulation of B cell-mediated diseases as well as allergic inflammation.

Acknowledgments

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Supplementary figures

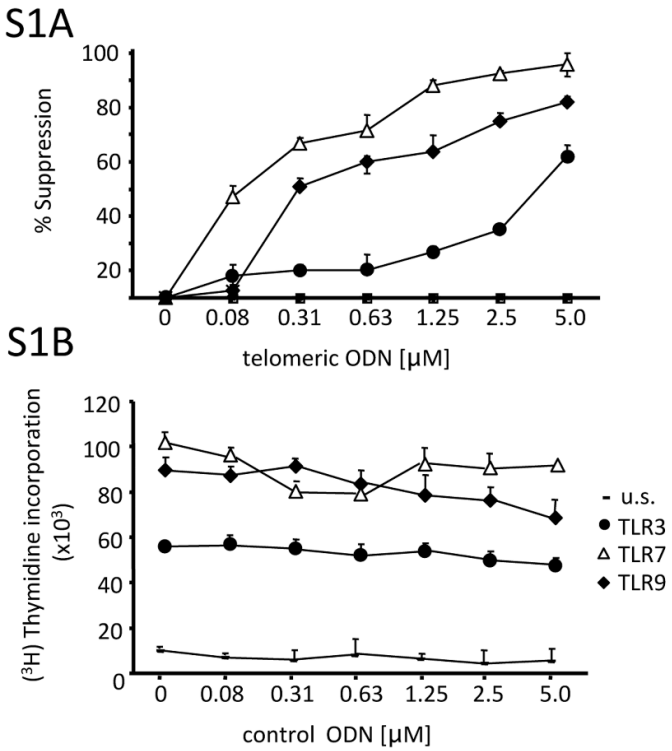

FIGURE S1. *Percentage suppression of TLR-induced PBMC proliferation at different concentrations of telomeric ODN. (A) One representative of 5 different experiments is shown and (B), control ODN.*

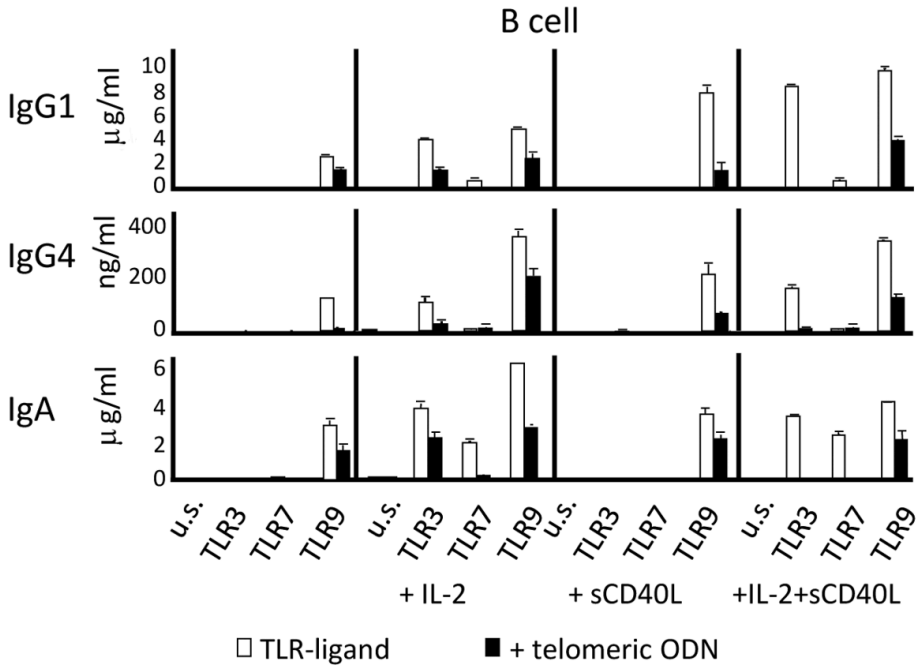

***FIGURE S2**. Different requirements in IgG1, IgG4 and IgA production by B cells with TLR3, 7 and 9 stimulation and their suppression by telomeric ODN. IgG1, IgG4 and IgA immunoglobulin synthesis by B cells 2x10^6/ml in 200 µl in 96-well plates was stimulated with TLR3, 7 and 9 ligands in the absence or presence of IL-2 and sCD40L or both. TLR7 and TLR9 ligands and telomeric ODN were used at 1 µM and TLR3 ligand was used at 25 ug/ml. IgG1, IgG4 and IgA were measured in day 12 supernatants. In order to examine TLR3, 7 and 9 mediated antibody secretion from pure B cells, highly pure CD19$^+$ B cells were used in stimulation assays. In contrast to PBMC, poly(I:C) and imidazoquinolone stimulation did not induce any antibody production by pure B cells, whereas stimulation with CpG 2006 was sufficient to induce IgG1, IgG4 and IgA production. The induction of IgG1, IgG4 and IgA by TLR3 and TLR7 stimuli was observed in the presence of IL-2. Similar to PBMC, stimulation of sCD40L alone did not show any additive effect in the absence of IL-2 on B cells, but significantly increased IgG1 production. Albeit at different suppressive levels, telomeric ODN significantly inhibited immunoglobulin synthesis by pure B cell cultures in all conditions tested.*

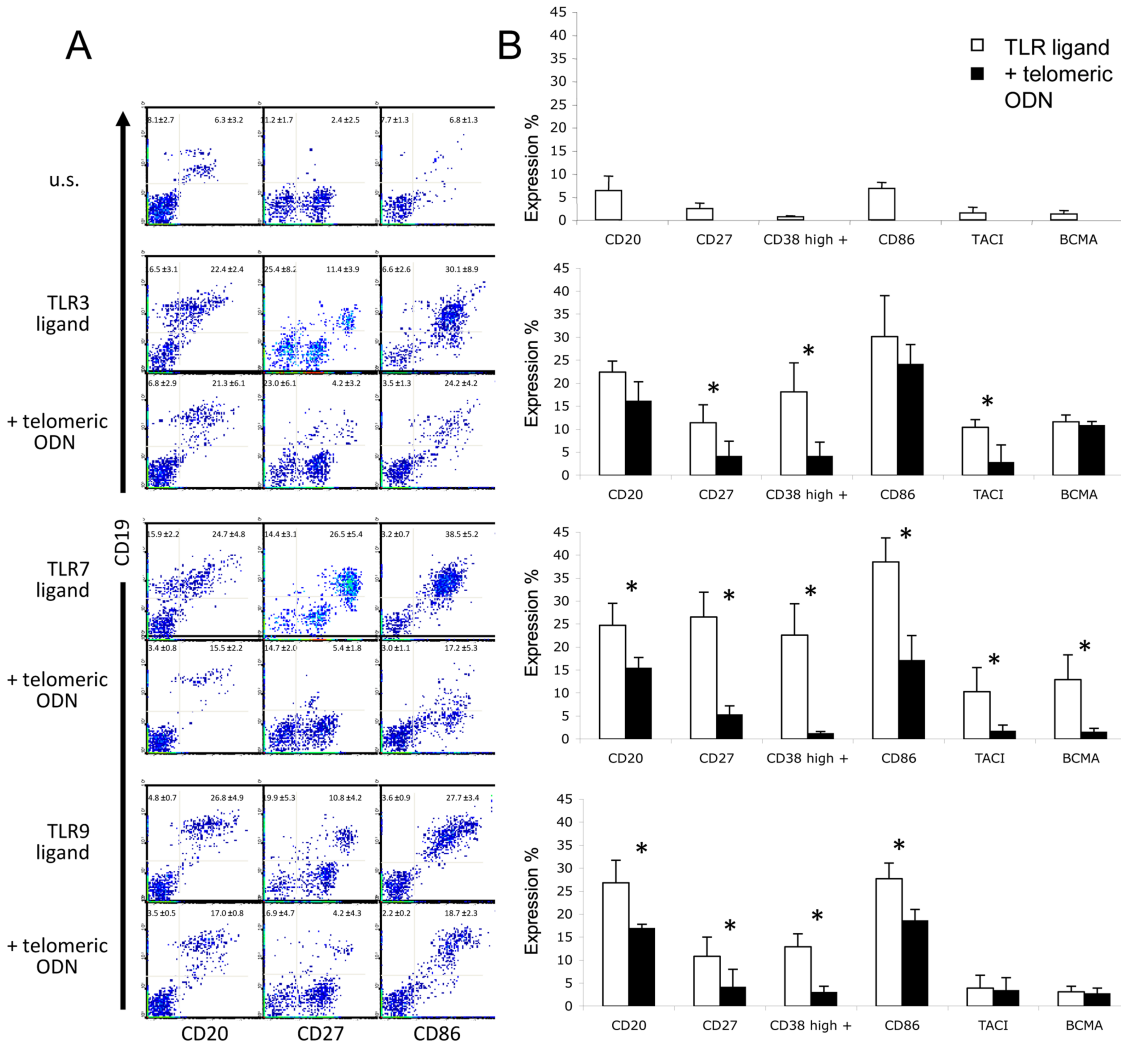

***FIGURE S3**. TLR3-, TLR7- and TLR9-ligand induced B cell differentiation and suppression by telomeric ODN. Human PBMC were stimulated with TLR3, 7 and 9 ligands in the presence of IL-2 with and without telomeric ODN for 6 days. Cells were stained with mAbs to CD19, CD27, CD38, CD80, CD86, BAFF-R, TACI and BCMA for flow cytometric analysis. (A) The gated area shows the percentage of positive cells for CD20, CD27 and CD86. Mean±standard deviation of percentage of surface expression in 2 different experiments is shown in each histogram. (B) Telomeric ODN suppressed $CD19^+CD27^+$, $CD19^+CD27^+$ in all conditions and $CD19^+CD20^+$, $CD19^+CD86^+$ cell proliferation stimulated with TLR7 and TLR9. One representative of 3 experiments is shown. *P<0.05.*

6.4 Contributions to publications

For the publication titled: “IgG4 production is confined to human IL-10-producing B regulatory cells that suppress antigen-specific immune responses”, I contributed to all experiments except the methodology of the whole genome microarray expression analysis.

For the publication titled: “Human IL-10-overexpressing B cells possess extensive regulatory capacity toward both innate and adaptive immune responses”, I contributed to the experiments depicted in figures 2, 3, 4 and 5.

For the publication titled: “Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligonucleotides”, I contributed to the experiments depicted in figures 2, 3, 4 and 6.

7 General Discussion

B cells play an essential role in the sensitization phase of allergic responses mainly by production of allergen-specific IgE antibodies. Th2 cells and their cytokines IL-4 and IL-13 play a key role in the induction of IgE. A physiologic way to dampen IgE-mediated allergic reactions is to skew allergen-specific IgE, towards allergen-specific anti-inflammatory IgG4. IL-10 can suppress allergic responses in several ways including suppression of antigen-presentation, suppression of T cell activation and suppression of IgE induction in B cells. On the other hand, IL-10 has been shown to enhance IgG4 production by B cells. Recent developments in the field of B cell biology have uncovered previously underappreciated functions of B cells that are primarily mediated by production of IL-10.

7.1 Human IL-10-producing Br1 cells are potent suppressors of antigen-specific T cell responses and selectively upregulate IgG4 production.

The primary aim of our study was to characterize human inducible IL-10-producing B cells. To study these cells, we used healthy donors, bee venom allergic patients that received bee venom SIT and healthy beekeepers who were exposed to high doses of bee venom. We applied the commonly used TLR9-L CpG2006 to induce IL-10 production in B cells and isolated IL-10-producing B cells using a cytokine secretion assay. This approach allowed the isolation of a pure population containing all B cells that produce IL-10 in response to this stimulus. Furthermore, because this system does not require permebealization of the cell membrane, viable cells could be isolated, which were used to perform functional experiments.

Phenotype of Br1 cells

In analogy to inducible IL-10-producing Tr1 cells, we designated these inducible IL-10-producing B cells as Br1 cells. Analysis of Br1 cells and non-IL-10-producing cells by gene arrays provided us with a list of differentially

expressed genes. These included among others surface markers and transcription factors. We have not succeeded to identify a master transcription factor that is specific for Br1 cells. Expression of CD25, CD71 and CD73 showed marked differences between both subsets and these differences could be confirmed by flow cytometry. Therefore these markers could be used as surrogate markers for isolation of Br1 cells. Indeed, sorting of resting $CD19^+CD73^-CD25^+CD71^+$ B cells followed by TLR9-L stimulation demonstrated that the most of the IL-10 production in response to this stimulation originated from this B cell population. We found marked differences regarding the phenotype of Br1 cells and the previously described $CD19^+CD24^{hi}CD38^{hi}$ human regulatory B cell population.[159] Br1 cells were not enriched among $CD19^+CD24^{hi}CD38^{hi}$ B cells but were equally distributed among $CD24^{int}CD38^{int}$, $CD24^{hi}$ $CD38^-$ and $CD24^{hi}$ $CD38^{hi}$ B cells. Furthermore, Br1 cells were not restricted to naïve or memory B cells since both $CD27^-$ and $CD27^+$ B cells produced IL-10 in response to TLR9-L stimulation. This suggests that there are multiple subsets of B cells that can produce IL-10 in response to distinct stimuli. $CD19^+CD24^{hi}CD38^{hi}$ represents a subset of B cells that produces IL-10 in response to CD40 ligation (a surrogate for T cell help) while $CD19^+CD73^-CD25^+CD71^+$ Br1 cells produce IL-10 in response to TLR9-L stimulation (representing microbial exposure). Similar to other proposed markers for isolation of IL-10-producing B cells this set of markers does not include all Br1 cells and cell sorting based on IL-10 expression remains the best strategy to purify the total Br1 population.

Function of Br1 cells in the regulation of T cell responses and the production of immunoglobulins

Regarding the function of Br1 cells we investigated two main directions: 1) Direct suppression of antigen-specific T cell responses and 2) Immunoglobulin production by Br1 cells. Co-cultures of Br1 cells with antigen-stimulated autologous PBMC demonstrated that Br1 cells are very potent suppressors of antigen-specific T cell proliferation that can inhibit up to 50% of

$CD4^+$ T cell proliferation at a ratio of 1 B cell to 25 responder cells. Similar findings have been reported using other regulatory B cell populations.

We were primarily interested in the isotype of immunoglobulins that are produced by Br1 cells. It has been previously demonstrated that IL-10 can augment IgG4 production in B cells while at the same it suppresses IgE.[63, 64] TLR9-L stimulation induced IgG1, IgG4, IgA and IgE production in purified peripheral $CD19^+$ cells from healthy donors. IL-10 had a selective synergistic effect on TLR9-L-induced IgG4 production while the production of other immunoglobulin isotypes was not increased. Most importantly, we found that Br1 cells, which secrete IL-10 in response to TRL9-L stimulation, show an increased expression of IgG4 at the mRNA level while expression of IgG1, IgA and IgE was not increased in Br1 cells. We then isolated IL-10-secreting Br1 cells from naïve TLR9-L-stimulated ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cell populations and measured the immunoglobulin isotype produced by these populations. IgG1, IgG4 and IgA were produced in all populations. The highest amounts of these immunoglobulins were produced by memory B cells (both Br1 and IL-10-negative cells), which was to be expected based on the fact that the $CD27^+$ B cell population includes the vast majority of IgG- and IgA-switched memory cells while $CD27^-$ B cells are primarily non-switched naïve B cells. Interestingly, there was a striking increase in the production of IgG4 by the $IL\text{-}10^+$ Br1 cells compared to the $IL\text{-}10^-$ cells that were isolated from the $CD27^-$ B cells. The absolute quantity of IgG4 that was produced $CD27^-IL\text{-}10^+$ Br1 cells was up to 6-fold lower than the amounts that were produced by both $IL\text{-}10^-$ and $IL\text{-}10^+$ $CD27^+$ B cells. But where IgG4 produced by $CD27^+$ B cells was most likely the result of differentiation of already switched memory B cells to plasma cells, the production of IgG4 in the $CD27^-IL\text{-}10^+$ Br1 cells suggests a switching event that is selectively occurring in naïve Br1 cells and specifically induces CSR from IgM to IgG4.

Induction of allergen-specific IgG4 is a hallmark for a healthy response to high-dose antigen exposure. Allergic individuals develop allergen-specific IgE

antibodies without a strong allergen-specific IgG4 response. Allergic patients who receive allergen SIT frequently develop allergen-specific IgG4 antibodies, which reach sufficiently high concentrations and/or affinities within 3 months to efficiently compete with IgE for allergen binding. This reduces binding of allergen to IgE and consequently can suppress IgE-FAP as well as mast cell and basophil degranulation.[76, 98] Both these mechanisms are important targets for intervention aimed at ameliorating allergic responses. Clearly, prevention of mast cell and basophil degranulation in response to allergen exposure will prevent the onset of immediate type hypersensitivity reactions. Prevention of IgE-FAP will suppress priming and reactivation of allergen-specific T cells.[98, 258, 259]

Increase in frequency of allergen-specific Br1 cells during SIT

A shift towards IL-10-producing allergen-specific Tr1 cells during tolerance induction has been reported in beekeepers.[102] Furthermore, healthy IL-10-producing Tr1 cells are the dominant allergen-specific Th cell subset in healthy individuals while IL-4-producing Th2 cells have a high frequency in allergic individuals.[101] In this study we determined the frequency of PLA-specific IL-10-secreting Br1 cells from allergic individuals before and after 110 days of ultra rush bee venom SIT and compared these frequencies to those observed in highly exposed healthy individuals (beekeepers). We found that the frequency of PLA-specific Br1 cells increased in patients receiving SIT and reached levels comparable to those observed in highly exposed healthy individuals. At the same time a strong decrease in the ratio of circulating PLA-specific IgG4 vs. IgE antibodies was observed.

Taken together, our data strongly supports a role for IL-10-producing Br1 cells in peripheral tolerance induction mediated by secretion of IL-10 and production of IgG4.

7.2 Characterization of IL-10-overexpressing B cells

In addition to the characterization of inducible IL-10-producing Br1 cells, we wanted to investigate direct effects of IL-10 expression in human B cells. Therefore we established and optimized a method for efficient transfection of human peripheral B cells to overexpress IL-10. Using the approach, we studied the effects of IL-10-overexpression on the phenotype and immunoregulatory potential of B cells on different types of immune responses.

Transfection of IL-10 gene into B cells led to rapid upregulation of expression of IL-10 and SOCS3, which is a direct IL-10-responsive gene and could serve as a control for overexpression of biologically active IL-10. Production of proinflammatory cytokines and chemokines were significantly decreased while concentrations of anti-inflammatory cytokines were significantly elevated in IL-10-overexpressing B cells. Furthermore, IL-10-overexpressing B cells showed upregulation of GARP and IL2RA expression, molecules that are associated with regulatory T cells. When compared to $CD25^{+}CD71^{+}CD73^{-}$ Br1 cells, IL10-overexpressing B cells express more CD25, while CD71 and CD73 were not affected by IL-10 overexpression. In addition CD5 expression showed a trend towards an increase on IL-10-overexressing B cells. These data suggest that IL-10 overexpression directly or indirectly induces CD25, and CD5 expression in human B cells. Furthermore, IL-10-overexpressing B cells downregulated activating co-stimulatory molecules (CD80 and CD86) while upregulating inhibitory PD-L1.

We did not observe a significant upregulation of IgG4 production in IL-10-overexpressing B cells. In fact, a modest suppression of IgE production was the only effect of IL-10 overexpression at the level of immunoglobulin production. This indicates that IL-10 in itself is not sufficient to induce IgG4 production (This can also be concluded from figure 4B of chapter 6.1).

IL-10-overexpressing B cells potently suppressed TLR2-L- and TLR4-L-induced proinflammatory cytokines and chemokines production by PBMC. This effect may result from the impact of IL-10-overexpressing B cells on

MDDC that extends from inhibition of cytokine release to suppression of their maturation downregulating their overall co-stimulatory potential (reduced CD80, CD86 and CD83, and induced PD-L1 surface expression) and therefore rendering MDDC toward a regulatory phenotype. Finally, IL-10 overexpressing B cells showed the capacity to potently suppress proliferation of antigen-stimulated PBMC cultures.

Taken together, IL-10 overexpression in B cells induces SOCS3, GARP and IL2RA expression all three molecules described to be highly expressed in regulatory T cells, along with increased CD1d, CD5 and CD38 expression and decreased capacity of co-stimulation. IL-10-overexpressing B cells are able to exert remarkable suppressive effect by acting directly on mechanisms of rapid-acting innate immunity through inhibiting the release of cytokines from TLR-L stimulated PMBC. Furthermore, IL-10-overexpressing B cells act on professional APCs by reducing their maturation profile resulting in reduction of cytokine production and co-stimulatory potential. IL-10-producing B cells are particularly potent in inhibiting antigen-specific proliferative response *in vitro* supporting the role of antigen specific IL-10 producing B cells limiting the memory T responses *in vivo*.

These findings confirm the importance of IL-10-producing B cells regulating different aspects of immune response, particularly their effect on dendritic cells and bring better understanding of the capacity of solely IL-10 on B cell physiology and regulatory profile applicable for their specific targeting in the fields of allergy and asthma, autoimmunity and tumor immunity.

7.3 Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligonucleotides.

Ligands for seven human TLRs were tested for their capacity to activate human peripheral B cells. TLR3-L, TLR7-L and TLR9-L induced proliferation of human B cells in whole PMBC. Only TLR9-L directly induced proliferation of

purified naïve and memory B cells while B cell activation by TLR3-L and TLR7-L was indirect and required the help of other cells, as purified B cells did not proliferate upon stimulation with these factors. Addition of IFN-α, IFN-β or pDCs to TLR7-L-stimulated B cells facilitated proliferation of purified naïve and, more pronouncedly, of memory B cells. The combination of TLR3-L with IFN-α or IFN-β induced detectable proliferation only in memory B cells. All these proliferative responses were suppressed by telomeric ODN.

IgG1, IgG4 and IgA production was induced in PBMC cultures upon stimulation with TLR3-L, TLR7-L or TLR9-L while only TLR9-L induced detectable amounts of these immunoglobulins in cultures with pure B cells. Addition of CD40L and IL-2 augmented TLR-L-induced immunoglobulin production in most cases. IgE could only be detected in PBMC cultures stimulated with CD40L + IL-4 with or without TLR-L. Telomeric ODN suppressed the production of all measured immunoglobulin isotypes. This suppressive effect may be attributed to reduced CSR, as the expression of the gene (*AICDA*) encoding a key enzyme in this process (activation-induced deaminase) was significantly reduced in the presence of telomeric ODN. Another level of suppression mediated by telomeric ODN was observed at the level of plasma cell differentiation. The frequency of plasma cells induced by TLR9-L stimulation was suppressed by approximately 50% in the presence of telomeric ODN.

These findings demonstrate that direct or indirect stimulation of human B cells through endosomal associated TLRs plays a pivotal role in polyclonal B cell activation and immunoglobulin production to maintain long-term serological memory in humans. Furthermore, these data reveal a direct and general suppressive effect of mammalian telomeric ODN on the activation of human B cells at the level of proliferation, CSR, plasma cell differentiation and immunoglobulin production. Suppression of B cell responses by telomeric ODN may represent a natural mechanism to control B cell activation and maintain immune homeostasis under chronic inflammatory conditions.

7.4 Conclusion and outlook

We conclude that the immunoregulation by B cells and the regulation of B cell responses have a pivotal role in the maintenance and induction of peripheral tolerance. This thesis demonstrates that human regulatory B cells can contribute to the control of immune responses in several ways: I) Direct suppression of antigen-specific T cell proliferation; II) production of anti-inflammatory IgG4 immunoglobulins, III) IL-10-mediated inhibition of pro-inflammatory cytokine production in response to TLR-triggering; IV) Suppression of maturation and antigen-presentation capacity of DCs. We also demonstrated that allergen-specific inducible IL-10-producing Br1 cells expand during SIT, which supports a role for these cells in the course of peripheral tolerance induction. The suppression of B cell responses by telomeric ODN provides a mechanism to prevent exaggerated B cell responses during chronic inflammation and as such can play a role in maintenance of peripheral tolerance.

Further characterization of human regulatory B cells and delineation of the mechanisms that play a role in their function may provide novel therapeutic options for the treatment of chronic inflammatory diseases.

8 Curriculum vitae

Personal data

Name:	VAN DE VEEN
First name	Willem Derk
Address	Dorfstrasse 29B CH-7260 Davos Dorf Switzerland
Date of birth	April 20, 1983
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Education

2008 - 2013	Employed as a PhD student at the Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland. Title: The Role of Human Regulatory B cells and B cell Regulation in Peripheral Tolerance.
2007	**Internship** at the protease platform of Novartis Institutes for BioMedical Research (NIBR), Basel, Switzerland.
2006	**Master thesis** at the department of allergology/dermatology, University Medical Center (UMC), Utrecht, the Netherlands. Title: A Role for Serum IgE-Facilitated Antigen Presentation in Cow's Milk Allergy?
2006 - 2008	**MSc in Biomedical Sciences** with specialization in Immunology and Infectious Diseases at University of Utrecht, the Netherlands. *Graduated 28-04-2008*
2001 - 2005	**BSc in Biology** with specialization in Cell Biology at University of Wageningen, the Netherlands. *Graduated 30-06-2005*
1994 - 2001	Atheneum (Dutch pre-university education) at C.C. de Noordgouw in Heerde, the Netherlands. *Graduated 15-06-2001*

Awards and honors

Travel grant and free registration for the 1st iFReC SIgN Winter School 2012 in Awaji Island, Japan

Travel grant and free registration for the 30th EAACI Congress 2011 in Istanbul, Turkey

Best presentation award at the SIAF science day, Davos, Switzerland, 14th December 2011

Best presentation award at the SIAF science day, Davos, Switzerland, 15th December 2010

Examinations and courses during PhD

Graduate Courses Life Science Graduate School Zürich (LSGSZ):

2010 – 2011	Immunology lectures SIAF
06 – 10 June 2010	MIM Introductory Block Course
11 – 12 March 2010	Cell Sorting Cours
23 – 24 November 2009	Introduction in Research Ethics & Biopatents

Other courses:

16-20 January 2012	1st IFReC-SIgN Winter School on advanced immunology, Awaji, Japan
04 – 06 September 2011	4th MIM Retreat, Chandolin, Valais, Switzerland
30 march – 1 April 2011	Wolfsberg Meeting, Schloss Wolfsberg, Switzerland
22 – 24 March 2010	Wolfsberg Meeting, Schloss Wolfsberg, Switzerland
11 – 14 February 2010	8th EAACI Immunology Winter School, Grainau, Germany
11 – 15 January 2010	Einführung in die Labortierkunde (BIO412)
05 – 07 November 2009	EFIS-EJI International Course on 'The Role of B cells in the Physiology and Pathology of the Immune System, Sorrento, Italy

Presentations in SIAF

Progress report	**Journal club**	**SIAF Science day**
09 December 2008	02 October 2008	16 December 2008
28 April 2009	03 March 2009	16 December 2009
20 October 2009	22 September 2009	15 December 2010
16 April 2010	29 June 2010	14 December 2011
12 October 2010	20 April 2011	20 December 2012
29 March 2011	27 March 2012	
05 October 2011	13 November 2012	
01 May 2012	09 April 2013	
23 October 2012		
28 May 2013		

Presentations at scientific congresses

2013	
23-26 June	EAACI-WAO World Allergy and Asthma Congress 2013 – Milan, Italy - Phenotypic and functional characterization of human allergen-specific memory B cells (Oral and poster presentation)
13-16 March	WIRM VII – Davos, Switzerland - Phenotype and function of human allergen-specific memory B cell subsets (Poster presentation)
14-15 January	MeDALL – Mechanisms of the Development of ALLergy - Third annual meeting and general assembly – Berlin, Germany - Characterization of human allergen-specific memory B cells in high-dose allergen-exposure models (Oral presentation)
2012	
16-20 January	1st IFReC-SIgN Winter School on advanced immunology – Awaji, Japan - Human Regulatory B cells Suppress Antigen-Specific Immune Responses and Produce IgG4 (Oral and poster presentation)
18-21 March	WIRM VI – Davos, Switzerland - Allergen-specific IgG4- and IgE-switched memory B cells in immune tolerance to allergens (Oral and poster presentation)
16-20 June	31st EAACI Congress – Geneva, Switzerland - Allergen-specific memory B cell subsets in immune tolerance to allergens (Oral presentation)
2011	
4-6 September	4th MIM retreat - Chandolin, Switzerland - Human Regulatory B cells in Allergic Disease. (Oral presentation).
11-15 June	30th EAACI Congress – Istanbul, Turkey - Human regulatory B cells suppress antigen-specific immune responses and give rise to IgG4-producing plasma cells. (Oral presentation).
30 march – 1 April	12th Meeting of the Swiss Immunology PhD students - Schloss Wolfsberg, Switzerland - Human IL-10-producing B cells negatively regulate immune responses and produce IgG4. (Oral presentation).
24-27 March	WIRM V – Davos Switzerland - Human IL-10-producing B cells suppress antigen-specific immune responses and produce IgG4. (Oral presentation).
2010	
18 May	Biorad bioplex user meeting - Basel Switzerland - Immune regulation by human IL-10-producing B cells. (Oral presentation).
22-24 March	11th Meeting of the Swiss Immunology PhD students - Schloss Wolfsberg, Switzerland - Immune regulation by human IL-10-producing B cells. (Oral presentation).

11-14 February	EAACI immunology winter school - Grainau Germany - Identification and Characterization of Human Regulatory B cells. (Oral presentation).
29 March - 1 April	WIRM IV - Davos Switzerland - Identification and Characterization of Human Regulatory B cells. (Oral presentation).

2009

5-7 November	B cell physiology and pathology of the immune system - Sorrento Italy - Human B regulatory cells, do they really exist? (Poster presentation).

Seminars

2011

16 June	Human regulatory B cells mediate immunological tolerance - Istanbul University, Istanbul, Turkey.
28 October	Human regulatory B cells and their protective potential against allergic disease - University Medical Center (UMC), Utrecht, the Netherlands.

Publications

Original articles:

IgG4 production is confined to human IL-10–producing regulatory B cells that suppress antigen-specific immune responses.
van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Söllner S, Akdis DG, Rückert B, Akdis CA, Akdis M.
J Allergy Clin Immunol. 2013 Apr;131(4):1204-12.

Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligodeoxynucleotides.
Sackesen C, van de Veen W, Akdis M, Soyer O, Zumkehr J, Rückert B, Stanic B, Kalaycl O, Alkan SS, Gursel I, Akdis CA.
Allergy. 2013 May;68(5):593-603.

Immune regulation by intralymphatic immunotherapy with modular allergen-translocation MAT vaccine
Anna Zaleska, Thomas Eiwegger, Özge Soyer, Claudio Rhyner, Michael B. Soyka, Cemalettin Bekpen, Duygu Demiröz, Angela Treis, Stefan Söllner, Oscar Palomares, Willem van de Veen, William W. Kwok, Horst Rose, Gabriela Senti, Thomas M. Kündig, Marek Jutel, Cezmi A. Akdis, Reto Crameri, Mübeccel Akdis
Submitted to J Allergy Clin Immunol.

Human IL-10-overexpressing B cells possess extensive regulatory capacity toward both innate and adaptive immune responses
Barbara Stanic, Willem van de Veen, Oliver Wirz, Beate Rückert, Stefan Söllner, Cezmi A. Akdis, and Mübeccel Akdis
In preparation

Review articles:

Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases.
Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, Klunker S, Meyer N, O'Mahony L, Palomares O, Rhyner C, Ouaked N, Schaffartzik A, van de Veen W, Zeller S, Zimmermann M, Akdis CA.
J Allergy Clin Immunol. 2011 Mar;127(3):701-21.e1-70.

Understanding the complexity of IgE-related phenotypes from childhood to young adulthood: a Mechanisms of the Development of Allergy (MeDALL) seminar.
Antó JM, Pinart M, Akdis M, Auffray C, Bachert C, Basagaña X, Carlsen KH, Guerra S, von Hertzen L, Illi S, Kauffmann F, Keil T, Kiley JP, Koppelman GH, Lupinek C, Martinez FD, Nawijn MC, Postma DS, Siroux V, Smit HA, Sterk PJ, Sunyer J, Valenta R, Valverde S, Akdis CA, Annesi-Maesano I, Ballester F, Benet M, Cambon-Thomsen A, Chatzi L, Coquet J, Demoly P, Gan W, Garcia-Aymerich J, Gimeno-Santos E, Guihenneuc-Jouyaux C, Haahtela T, Heinrich J, Herr M, Hohmann C, Jacquemin B, Just J, Kerkhof M, Kogevinas M, Kowalski ML, Lambrecht BN, Lau S, Lødrup Carlsen KC, Maier D, Momas I, Noel P, Oddie S, Palkonen S, Pin I, Porta D, Punturieri A, Rancière F, Smith RA, Stanic B, Stein RT, van de Veen W, van Oosterhout AJ, Varraso R, Wickman M, Wijmenga C, Wright J, Yaman G, Zuberbier T, Bousquet J; WHO Collaborating Centre on Asthma and Rhinitis (Montpellier).
J Allergy Clin Immunol. 2012 Apr;129(4):943-54.e4.

TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection.
Akdis M, Palomares O, van de Veen W, van Splunter M, Akdis CA.
J Allergy Clin Immunol. 2012 Jun;129(6):1438-49; quiz1450-1.

Natural Killer cells in Allergic Diseases
Günnur Deniz, Willem van de Veen, Mübeccel Akdis
J Allergy Clin Immunol. 2013 Sep;132(3):527-35.

Book chapters:

Induction of IgE Synthesis: Cellular Interactions and Molecular Events
Willem van de Veen (*in press)* Encyclopedia of Medical Immunology (I. MacKay & N. R. Rose, Eds.). New York: Springer.

Specific IgE
Willem van de Veen (*in press)* Encyclopedia of Medical Immunology (I. MacKay & N. R. Rose, Eds.). New York: Springer.

IgE isotype switching
Willem van de Veen (*in press)* Encyclopedia of Medical Immunology (I. MacKay & N. R. Rose, Eds.). New York: Springer.

IgE receptor
Willem van de Veen (*in press)* Encyclopedia of Medical Immunology (I. MacKay & N. R. Rose, Eds.). New York: Springer.

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